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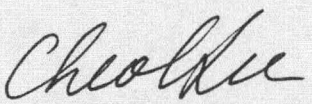

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THE INFLUENCE OF INTRINSIC AND EXTRINSIC FACTORS ON NEUROGENESIS

by
Cheol Lee

Doctoral Dissertation submitted to the faculty of the Graduate Program in Neuroscience of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 2008

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ABSTRACT

The Influence of Intrinsic and Extrinsic Factors on Neurogenesis

by

Cheol Lee

Neurogenesis is a complex process through which new neurons are generated. The process is regulated by combination and coordination of cell intrinsic and extrinsic factors. I analyzed the expression and molecular function of an intrinsic factor, the nuclear regulatory protein A + U-rich element-binding factor 1 (AUF1) in the developing and adult brain, and the role of an extrinsic factor, the vascular endothelial growth factor (VEGF) and its receptor Flk1 in mediating *de novo* neurogenesis in the injured adult brain.

The AUF1 is a known regulator of messenger RNA stability and also acts as a transcription factor upon binding to AT-rich DNA elements. Here I show that AUF1 is specifically expressed in subsets of proliferating neural precursors and differentiating postmitotic neurons of the developing cerebral cortex. Importantly, AUF1 was coexpressed with histone deacetylase 1 (HDAC1) and metastasis-associated protein 2 (MTA2), members of the nucleosome remodeling and histone deacetylase complex. AUF1 specifically and simultaneously bound to HDAC1, MTA2, and an AT-rich DNA element. These interactions have functional significance because AUF1's gene regulatory function was modulated by the extent of histone acetylation and, in cells lacking AUF1, the composition of the complex was modified. AUF1 was also expressed in *de novo* neurons in the adult dentate gyrus (DG). In AUF1 mutant animals, the size and cytoarchitecture of the DG were altered and the number of proliferating cells in the

DG was reduced, suggesting that AUF1 is a regulator of neurogenesis both in the developing and adult brain.

VEGF is a well-known angiogenic factor and also has neurotrophic effect. In primary embryonic neuronal cultures, Flk1 was expressed in proliferating cells and young neurons, and VEGF had both proliferative and antiapoptotic effects. VEGF receptor Flk1 was expressed by both neuroblasts and by maturing granule neurons in the normal adult DG. The expression of VEGF, but not that of Flk1, was significantly and specifically upregulated in the ipsilateral DG in the injured rat brain. To directly test the role of VEGF and Flk1 in regulating *de novo* hippocampal neurogenesis, recombinant VEGF or SU5416, an inhibitor to Flk1, was delivered into the ipsilateral cerebral ventricle of injured animals. VEGF infusion significantly increased the number of *de novo* granule neurons in the DG but not proliferating cells, suggesting that VEGF in the injured adult brain acts predominantly as a differentiation / survival factor. Interestingly, chronic treatment with SU5416 failed to lower the number of *de novo* neurons below control levels in the injured brain, suggesting that the observed effect of VEGF may involve mechanisms other than Flk1 activation.

Overall, we found that AUF1 is involved in regulating cortical neurogenesis in the developing brain through interactions with chromatin-remodeling molecules. This suggests a novel role for this already multifunctional molecule. In addition, I have shown experimental evidence that AUF is also involved in adult hippocampal neurogenesis. I found that VEGF is a mediator of *de novo* neurogenesis in the injured adult brain and its effect is likely similar to the developing brain. These findings suggest that the regulatory process on neurogenesis by intrinsic and extrinsic factors is conserved between the developing and adult brain. These results will help

to identify the genetic and environmental risk factors leading to developmental brain disorders and to develop treatment options to alleviate the consequences of brain injury.

LIST OF ABBREVIATIONS

5'Bio-rAT	biotinylated AT-rich DNA probes
ARE	A+U-rich elements
AUF1	A + U-rich element-binding factor 1
bHLH	basic helix-loop-helix
BrdU	bromo-deoxy uridine
CP	cortical plate
DAPSTER	DNA affinity preincubation specificity test of recognition
DG	dentate gyrus
dpi	days post injury
ds rAT	double strand AT-rich DNA probes
dsDNA	double stranded DNA
FACS	fluorescence-activated cell sorting
GCL	granule cell layer
HAT	histone acetyltransferase
HDAC	histone deacetylase
hnRNPD	heterogeneous nuclear ribonuclear protein D
LFP-TBI	lateral fluid percussion model of traumatic brain injury
MEF	mouse embryonic fibroblast
MTA2	metastasis associated protein 2
MZ	marginal zone
NEP	neuroepithelial stem cell

NSC	neural stem cell
NuRD	nucleosome remodeling and histone deacetylase
PP	preplate
RG	radial glial cell
RLU	relative light units
SGL	subgranular layer
SVZ	subventricular zone
TBI	traumatic brain injury
TSA	trichostatin A
UTR	untranslated region
VEGF	vascular endothelial growth factor
VZ	ventricular zone

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CHAPTER I

INTRODUCTION

Neurogenesis

Neurogenesis is a complex process through which new neurons are generated. Neurogenesis in a broader sense includes not only the proliferation of neural stem / progenitor cells but also the differentiation and selective elimination of cells by apoptotic cell death. It is believed that neurogenesis is regulated by two sets of factors, *i.e.* cell intrinsic factors and cell extrinsic factors. Intrinsic factors are programmed inside of a cell and regulate gene expression in a cell-autonomous manner. Intrinsic factors include a wide array of genetic information including transcription factors. Extrinsic factors are signals derived from outside of a cell. Extrinsic factors include soluble and solid-phase ligands, cell-cell contacts, and physical / biochemical properties of the local environment. Although much has been learned about the identities of intrinsic and extrinsic factors, we still lack a complete understanding of their respective contribution to the regulation of the developmental process. One of the long held dogmas of neuroscience was that neurogenesis only occurs during development. Studies have now clearly demonstrated that new neurons are generated throughout life in the adult mammalian brain. Importantly, adult *de novo* neurogenesis is upregulated after brain injury, suggesting that adult neurogenesis is an important component of the innate regenerative capacity of the adult brain. Despite its potential clinical relevance and the substantial progress made during the last few years, the full identity of molecules that mediate *de novo* neurogenesis in the injured adult brain is currently not known.

A better understanding of the identity of molecules that regulate developmental and adult neurogenesis will provide better insight into the cause of developmental brain disorders and will help in the development of therapeutic strategies aimed to alleviate the consequences of neuronal loss due to brain injury.

The specific aim of this thesis work was to provide better understanding of the role of intrinsic and extrinsic factors in *de novo* neurogenesis through investigating the molecular function of an intrinsic factor, the nuclear regulatory protein AUF1, in the developing brain (Chapter II) and in the adult brain (Chapter III), and to determine the role of an extrinsic factor, the growth factor VEGF, and its receptor Flk1 in mediating *de novo* neurogenesis in the injured adult brain (Chapter IV).

In the Introduction, I provide an overview of neurogenesis in the developing and in the adult brain. Then I briefly review factors that are involved in regulating the process. Lastly, I introduce two particular molecules AUF1 and VEGF, and describe their known functions and involvement in regulating neurogenesis.

Neurogenesis in the Developing Brain

During development, three distinct populations of neural stem / progenitor cells generate neurons (glial cells as well) in the embryonic telencephalon (Figure I-1). The first neural stem cells (NSCs), appearing at E10.5 in the developing rat brain, are the neuroepithelial stem cells (NEPs) that comprise the wall of the neural tube [1]. At the onset of neurogenesis at E12 in mice, NEPs located in the ventricular zone (VZ) either divide symmetrically to generate two new NEPs, or divide asymmetrically to generate a daughter NEP and either a neuron or a basal progenitor cell [2]. Newborn neurons then migrate to the preplate (PP) and basal progenitors

divide symmetrically to generate two neurons. As neurogenesis continues, NEPs become a distinct cell type called radial glial cell (RG). These cells have features of neural stem / progenitor cells and also astroglia. RGs divide either symmetrically to generate two identical daughter cells, or asymmetrically to generate an RG and either a postmitotic neuron or a basal progenitor cell. Newborn neurons at this stage migrate radially toward the cortical plate and form the distinct cortical layers in an 'inside-out' manner [3,4]. While RGs remain in the VZ, basal progenitor cells migrate outward and eventually form the subventricular zone (SVZ). These cells, also referred as intermediate progenitors, divide symmetrically to generate two neurons and may give rise to the majority of cortical projection neurons [5,6]. Most excitatory neurons of the adult neocortex are derived from neuroepithelial cells of the ventricular zone of the embryonic brain, whereas most cortical interneurons in rodents originate from the ganglionic eminence of the ventral telencephalon and migrate tangentially to the cortical plate [7].

Neurogenesis in the Adult Mammalian Brain

One of the central dogmas in neuroscience was that no neurons are generated in the adult brain. Studies, however, have demonstrated that there are neural stem / progenitor cells in the adult mammalian brain, including the human brain, and these cells can give rise to new neurons and glial cells throughout life [8]. These adult neural stem / progenitor cells show many of the characteristics of neural stem / progenitor cells in the developing brain. Similarly to their embryonic counterparts, they are self-renewing and multipotent.

Neurogenesis in the adult brain, however, is much more restricted than in the developing brain. There are only two major neurogenic regions in the adult mammalian brain, the SVZ of the lateral ventricle and the subgranular layer (SGL) of the dentate gyrus (DG). It should be

noted that the adult cortex is also capable of generating new neurons that have been found in the prefrontal, inferior temporal, and posterior parietal cortex of adult macaques and in the anterior neocortex of adult rodents [9]. Although new cortical neurons that were born after targeted neuronal death can form long-distance corticothalamic connections [10], most, if not all, of new neurons in the adult neocortex have features of interneurons rather than pyramidal cells [11]. The extent of this cortical neurogenesis is quite limited and the number of new neurons is very low compared to the two major neurogenic regions.

Neural stem / progenitor cells in the adult SVZ of the lateral ventricle constantly proliferate and migrate along the rostral migratory stream toward the olfactory bulb. They will eventually differentiate into granule cells and interneurons of the olfactory bulb [12,13]. Neural stem / progenitor cells in the SGL of the DG proliferate and migrate short distances into the granule cell layer (GCL) (Figure I-2). Most of the newborn cells differentiate into dentate granule neurons and finally incorporate into hippocampal circuitry [14-18]. Because "neurogenesis" is a multi-step process, there is a heterogeneous population of newborn cells in the SGL and GCL. These cells range from proliferating stem / progenitor cells to fully differentiated *de novo* neurons. Cells in different developmental phases have been classified into six distinct stages based on their morphology and expression of markers (Figure I-3) [19]. Young neurons start to express postmitotic neuronal markers and project axons into the CA3 region as rapidly as 4-10 days after mitosis [19,20]. During this time window, the number of newborn cells decreases substantially, implying that some cells are selectively eliminated by apoptotic cell death [21]. The apical dendrites of newborn neurons reach the molecular layer and elaborate dendritic processes 10-14 days after birth [22]. Approximately 4 weeks after birth, *de novo* neurons show the morphological and electrophysiological features of mature neurons and

express markers of the mature phenotype. Importantly, they integrate into the hippocampal circuitry and become fully functional members of the neuronal circuitry [23].

Factors Controlling Neurogenesis in the Developing Brain

Neural stem / progenitor cells in the developing mammalian cortex have, to some extent, a default intrinsic program for birth order. Cell culture experiments have shown that embryonic neural stem / progenitor cells give rise to neurons first and glia later [24]. In addition, it has been shown that there is a sequential production of cortical neurons, *i.e.* deep layer neurons are generated first followed by upper layer neurons [25]. These results suggest that the timing for the normal order of cell-type generation is, at least in part, programmed within individual stem / progenitor cells by cell intrinsic factors also called intrinsic molecules. These molecules are mainly transcription factors and several of them have been identified. Proneural basic helix-loop-helix (bHLH) transcription factors have been shown to regulate multiple aspects of neurogenesis that include fate determination and neuronal differentiation [26]. The bHLH transcription factors Mash1 (mammalian achate-schute homolog 1) and Ngn1 (Neurogenin 1) are expressed in early stage embryos and thought to act as neuronal determination factors [27,28]. Neurogenic differentiation factors such as NeuroD are expressed later than the determination factors and they regulate neuronal development in postmitotic cells [26]. There are also negative regulators of neurogenesis such as the mammalian Hes1 (hairy and enhancer of split homolog 1) that inhibit neuronal differentiation through maintaining neural precursor cells in an undifferentiated state [29]. A good example of a non-bHLH transcription factor that is critical in neurogenesis is Pax6 (paired box 6). Pax6 is specifically expressed by RGs in the mouse dorsal telencephalon and in Pax6-deficient mice the cortical RGs have alterations in their morphology, number, molecular

phenotype, and cell cycle [30]. In *Small-eye* (Sey) mice, which lack functional Pax6, the cortex and cortical plate are thinner than those of wild type and the number of neurons in the Pax6 mutant cortex *in vivo* is reduced [31].

In addition to intrinsic factors, the process of neurogenesis is also regulated by extrinsic factors such as soluble growth factors. Their role in regulating the process is complex as most growth factors display multiple effects dependent on the cell population as well as on developmental stages. Moreover, the effect of a growth factor is typically mediated by particular tyrosine kinase receptors that can activate several different converging and diverging intracellular signaling pathways. Thus, the effect of soluble factors in controlling neurogenesis in the developing brain is likely accomplished by integrating multiple signals. One of the best understood factors is fibroblast growth factor 2 (FGF2). The primary function of FGF2 in the developing rodent brain is to promote proliferation of neural stem / progenitor cells [32]. FGF2 is also involved in cell fate determination of cortical progenitors *in vitro*, acting in a concentration-dependent manner. In low FGF2 concentration, only neuronal progeny is generated whereas glial progeny is produced from multipotent neural stem / progenitor cells in high levels of FGF2 [33]. Epidermal growth factor (EGF) acts mostly as a mitogen for telencephalic progenitor cells. Its effect is modulated by the expression level of EGF receptor (EGFR) by individual cells. EGFR is asymmetrically distributed in the two daughter cells and these cells can respond differently to EGF [34]. The copy number of EGFR can affect their proliferation, migration, and fate determination [35]. Another extrinsic molecule is platelet-derived growth factor (PDGF) that promotes neuronal differentiation of neural stem cells *in vitro* [36]. In order to survive after target innervation, postmitotic neurons require another set of extrinsic factors. Many of these are

so-called neurotrophic factors such as members of the neurotrophin family that are produced by the target cell or target tissue [37].

Epigenetic regulation has emerged as an important mechanism controlling gene expression during neurogenesis [38,39]. Epigenetics refers to alterations in gene expression that are controlled by heritable but potentially reversible modifications in DNA and / or chromatin structure without changes in the underlying DNA sequence of the organism [40]. For example, DNA binding protein Repressor Element 1-Silencing Transcription Factor (REST) represses expression of neuronal genes containing the Repressor Element 1 (RE1) motif. REST exerts its function by interacting with corepressors that recruit histone modifying enzymes such as histone deacetylases (HDACs) and a histone methyltransferase. During neurogenesis the function of REST is downregulated, resulting in the development of various neuronal phenotypes [41]. It has been shown that inhibition of HDAC promotes neuronal differentiation of neural stem / progenitor cells through the induction of neurogenic transcription factor NeuroD [38].

Factors Controlling Neurogenesis in the Adult Brain

It has been estimated that up to 9,000 new cells are produced each day in the DG of the adult rat. Although many of them die by apoptotic cell death, the majority of the surviving cells will differentiate into neurons [42]. Just as in the developing nervous system, neurogenesis in the adult DG is controlled by both intrinsic and extrinsic factors [43,44]. While some intrinsic factors regulating adult neurogenesis are distinct from embryonic neurogenesis, some factors have conserved regulatory mechanism [45,46].

Known extrinsic factors that regulate adult neurogenesis include neurotrophic factors such as insulin-like growth factor I (IGF-I) [47], FGF-2 [48], and also functional changes such as

hippocampus-dependent learning [49], an enriched environment [50], and voluntary physical exercise [51]. Importantly, several pathological conditions that are associated with neuronal loss such as ischemia [52], seizure [53], excitotoxicity [54] and traumatic brain injury [55,56] also significantly increase the rate of *de novo* neurogenesis after a latent period (Table I-1). These findings suggest that *de novo* neurogenesis may be a part of the regenerative process as the injured adult brain attempts to replace lost neurons.

My thesis investigates the roles of AUF1 as an example of an intrinsic factor in regulating neurogenesis in the developing brain and VEGF as an example of an extrinsic factor in regulating adult hippocampal neurogenesis after traumatic brain injury.

AUF1

A+U-rich element-binding factor 1 (AUF1) is also known as heterogeneous nuclear ribonuclear protein D (hnRNPD) that belongs to the functionally diverse hnRNP family. AUF1 has four isoforms (p37, p40, p42, and p45) generated from the primary transcript through alternative splicing and each isoform has distinct biological functions. AUF1 was originally identified as a protein that binds to A+U-rich elements (AREs) of the 3' untranslated region (UTR) of mRNAs [57]. *In vitro* studies have shown that AUF1 is a key regulator of mRNA stability by degrading mRNAs with AREs as a post-transcriptional regulation of gene expression. AUF1-dependent mRNA degradation is closely linked to the ubiquitination and proteasome targeting of AUF1 [58], and to the association of AUF1 with the exosome, an important RNA degradation machinery in the cell [59]. AREs are commonly found within the 3' UTRs of labile, or short lived, mRNAs such as proto-oncogenes [60], growth factors [61], cytokines and chemokines [62], and cell cycle regulatory genes [63].

Besides the ability to associate with mRNA stabilization, AUF1 has also been described to participate in other regulatory mechanisms. For example, p40 isoform has been shown to interact with the TATA-binding protein and can activate reporter gene expression in a cell culture model [64]. The p42 isoform binds single-stranded telomere DNA and is involved in telomere maintenance [65,66]. Our previous study showed that AUF1 binds the AT-rich double stranded DNA (dsDNA) *cis*-regulatory element and acts as a transcriptional regulator [67].

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) was first described as a vascular permeability factor, then as a major angiogenic factor which promotes proliferation and survival of vascular endothelial cells and vascular formation [68]. VEGF is essential for vasculogenesis during development and even heterozygous VEGF-deficient mice die in midgestation due to abnormal development of blood vessels [69]. There are several homologues of VEGF and the exact term should be VEGF-A, but according to convention I will refer to VEGF-A simply as VEGF. The VEGF gene consists of 8 exons and 7 introns in human and rodents [70]. The active form of VEGF protein is an approximately 45 kDa homodimer linked by disulfide bonds. There are five isoforms due to alternative splicing. $VEGF_{165}$ is the predominant form and shows most active biological function in human. In rats, it is $VEGF_{164,}$ which is one amino acid shorter than that of human. VEGF belongs to a growth factor superfamily that includes the PDGF, placenta growth factor (PlGF), and the VEGF homologues VEGF-A through D.

Because of its significance, research has primarily focused on the role of VEGF in vascular endothelial cell development and VEGF-induced angiogenesis during the development of cancer. However, an increasing amount of evidence has suggested that VEGF also has

neurotrophic and neuroprotective effects [71-74]. Treatment of primary neuronal cultures with VEGF caused neurotrophic effects such as neurite outgrowth [75]. When VEGF is injected directly into the adult brain, it stimulates neurogenesis in the hippocampus [76]. VEGF has also been shown to mediate the positive effects of enriched environment and exercise, which both increase the rate of adult *de novo* neurogenesis [77,78]. Intraventricular administration of recombinant VEGF had a neuroprotective effect on motorneurons in a rat model of amyotrophic lateral sclerosis (ALS) [79].

VEGF receptors and signaling

Three VEGF receptors have been identified so far: VEGFR-1 (Flt1), VEGFR-2 (Flk1 or KDR), and VEGFR-3. All of them are membrane-bound receptor tyrosine kinases that have structural similarities [80,81]. Flt1 deficient mice are embryonic lethal primarily due to dysregulated proliferation of endothelial progenitors [82]. Because there is a soluble form of Flt1, it has been proposed that Flt1 may serve as a "decoy" receptor that sequesters VEGF to prevent excessive activation of Flk1 by soluble VEGF [83]. The function of Flt1, however, is still not well established. There are also other VEGF receptor types such as VEGFR-3 that is expressed predominantly in the lymphatic vasculature and a coreceptor neurophilin [84,85].

Flk1 has been known to transduce most of the VEGF-induced effects including its neuronal effects [86]. For example, the neurotrophic effect of VEGF is inhibited by Flk1 antisense oligodeoxynucleotides treatment, but not by antisense Flt-1 [75]. Also, axonal outgrowth is blocked by a specific antagonist to Flk1 [74]. Flk1 consists of a seven immunoglobulin-like extracellular domain, a single transmembrane domain, a receptor tyrosine kinase domain, and a recruiting domain which contains Tyr residues interacting with other

intracellular signaling molecules [81]. VEGF binding to Flk1 induces, like many other receptor tyrosine kinases, the dimerization and activation of the receptor by autophosphorylation. Subsequently, multiple Tyr residues in the intracellular domain of Flk1 become phosphorylated and serve as docking sites for cytoplasmic signaling molecules [87]. Several signaling pathways that mediate VEGF/Flk1 signaling have been identified. Neuroprotection by VEGF in the hypoxic / ischemic brain may be associated with activation of Flk1, followed by the activation of phosphoinositide-3 kinase/protein kinase B (PI3K/Akt) anti-apoptotic pathway [88] and the subsequent inhibition of caspase-3 activity [89]. It has been also shown that VEGF-induced activation of mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) signaling pathway is involved in protection of cultured neurons from glutamate-induced toxicity [90] and proliferation of retinal progenitor cells [91].

Traumatic Brain Injury

Neurons in the adult mammalian brain are post-mitotic, terminally differentiated cells, so neuronal loss has especially severe functional consequences. Traumatic brain injury (TBI) is a significant cause of death and life-long disability [92]. The Centers for Disease Control and Prevention (CDC) has estimated that each year approximately 1.5 million Americans suffer from TBI, among whom, approximately 230,000 are hospitalized [93]. Approximately 50,000 Americans die each year following TBI, representing one-third of all injury-related deaths [94]. TBI is a leading cause of combat casualties within the military as well. In Desert Storm, up to 20% of combat wounds involved the head [95,96]. The importance of TBI lies not only on the high mortality and occurrence but also on those individuals who suffer permanent disabilities.

Although continuous education has helped to reduce the rate of TBI incident, currently no effective therapy is established to alleviate the disabilities resulting from TBI.

Research has shown that various forms of TBI frequently result in hippocampal damage leading to impaired hippocampus-dependent functions such as learning and memory. The response of the brain to trauma is complex and often associated with various biochemical and physiological changes. Interestingly TBI increases the rate of *de novo* hippocampal neurogenesis after a latent period, which is thought as an endogenous repairing mechanism after brain injury [55,56]. Although this phenomenon raises the hope that the injured brain can self-repair, the process is not sufficient for full recovery and the identity of molecules involved in regulating the process is currently not fully known.

Animal models of TBI

One of the best-established animal models of TBI is the lateral fluid percussion model of traumatic brain injury (LFP-TBI). Although LFP-TBI was initially designed to simulate the coup-contrecoup or the acceleration / deceleration injury in small animals, the pathophysiology of LFP-TBI indicates that it is rather a mixed model of TBI, *i.e.* it features both focal and diffuse brain injury. Experimental fluid percussion models produce brain injury by rapidly injecting fluid volumes into the closed cranial cavity. As such, the LFP-TBI produces rapid displacement of the cortex, a global increased intracranial pressure, subdural hematoma, subarachnoid hemorrhage, and white matter tears [97]. Since this LFP-TBI rat model was developed, numerous physiological, histopathological, and behavioral studies have shown that this is an extremely useful experimental model to study of the pathophysiology of TBI [98,99].

Summary

Neurogenesis in the developing brain and in the adult brain is influenced by various intrinsic and extrinsic factors in a cooperative and coordinated manner. The overall aim of this thesis work is to understand the role of two regulatory molecules, a nuclear protein and a soluble factor, on neurogenesis. The primary objectives in pursuit of the overall aim are to determine the role of nuclear regulatory protein AUF1 in the developing brain (Chapter II) and in the adult brain (Chapter III), and to determine the role of growth factor VEGF and its receptor Flk1 in regulating *de novo* neurogenesis in the adult brain after TBI by using the LFP-TBI model (Chapter IV). This information will contribute to understanding how intrinsic factors are involved in regulating neurogenesis in the developing and adult brain, and to the development of therapeutic strategies aimed to improve regeneration and restore functionality after neuronal loss due to neurotrauma.

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CHAPTER I Figures

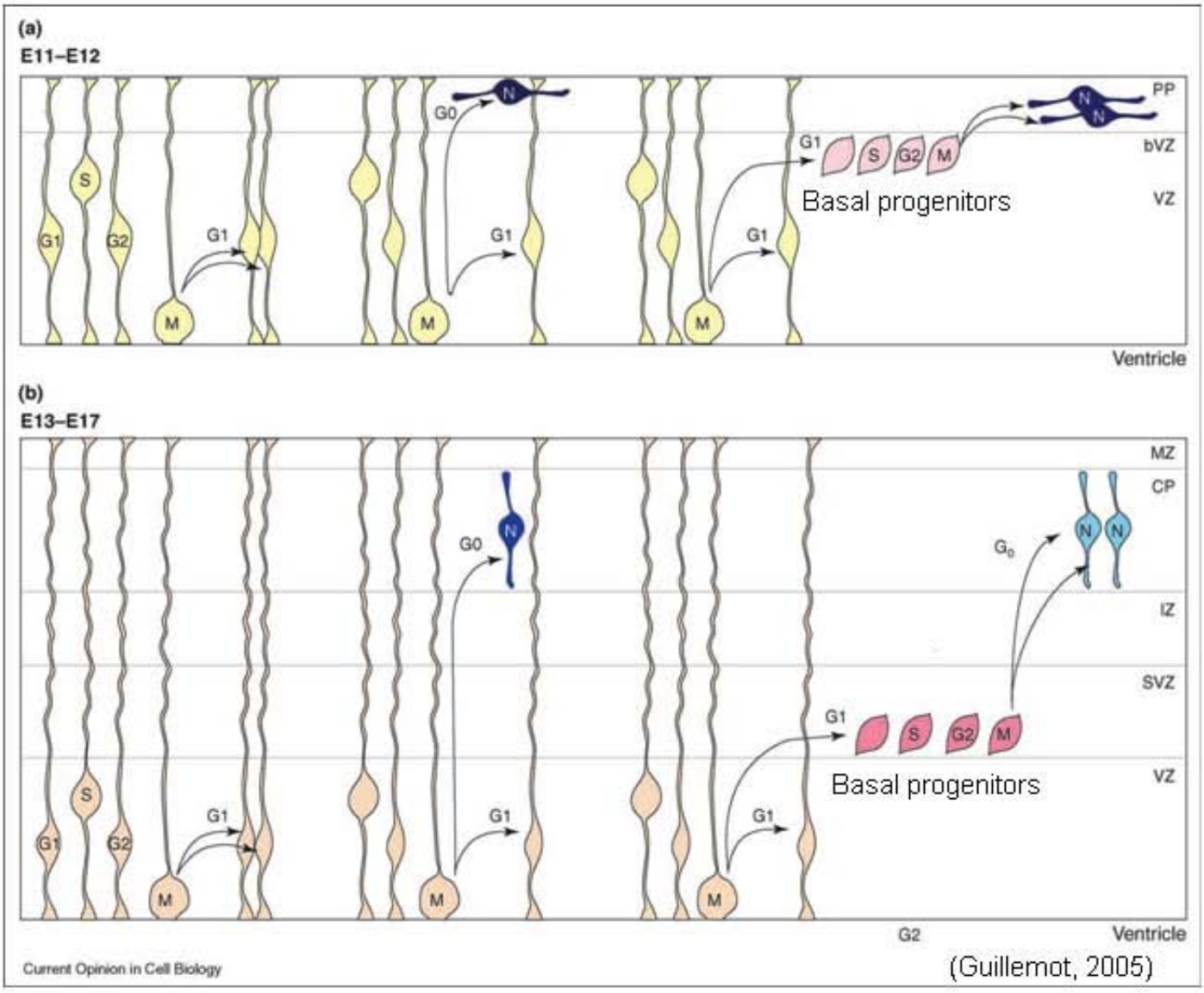

Figure I-1. Several types of progenitors contribute to neurogenesis in the developing mammalian cortex. (a) Neurogenesis from neuroepithelial stem cells (NEPs) in early embryonic stage. NEPs located in the ventricular zone (VZ) either divide symmetrically to generate two new NEPs, or divide asymmetrically to generate a daughter NEP and either a neuron or a basal progenitor cell. Newborn neurons then migrate to the preplate (PP) and basal progenitors divide symmetrically to generate two neurons. As neurogenesis continues, NEPs become radial glial cells (RGs). (b) Neurogenesis from RGs in late embryonic stage. RGs divide either symmetrically to generate two identical daughter cells, or asymmetrically to generate an RG and either a postmitotic neuron or a basal progenitor cell. Newborn neurons at this stage migrate radially toward the cortical plate (CP). G0, G1, S, G2, and M represent the phase of cell cycle. N, neuron; PP, preplate; bVZ, basal side of the VZ; MZ, marginal zone; IZ, intermediate zone; SVZ, subventricular zone

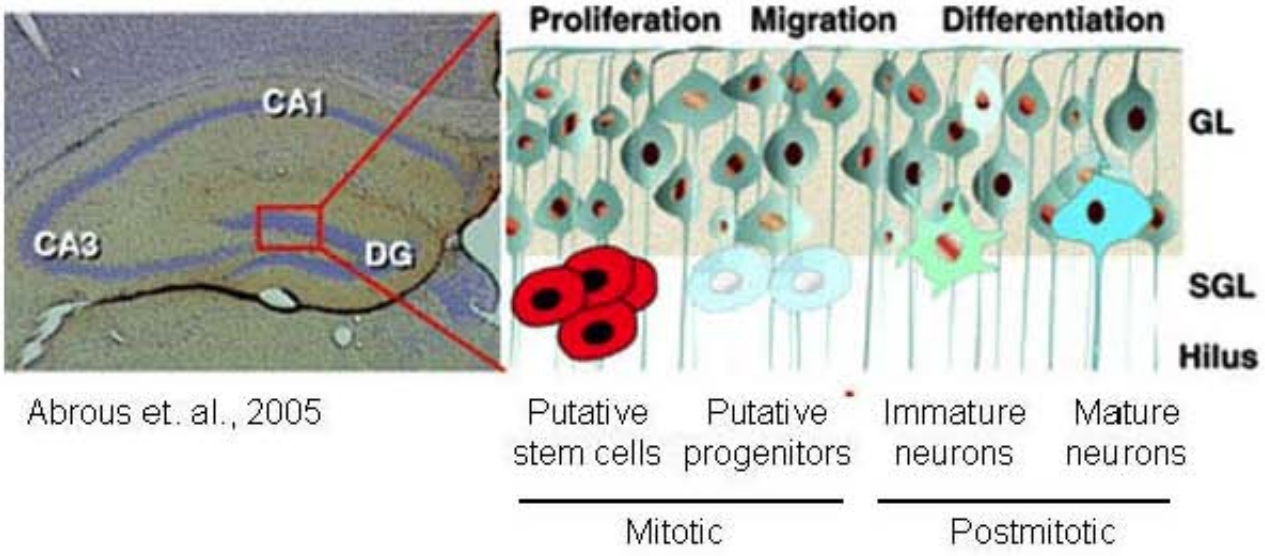

Figure I-2. Neurogenesis in the adult dentate gyrus (DG). Cells proliferate in the subgranular layer (SGL) and migrate to the granule cell layer (GL), and they differentiate mostly into granule cells. Cells in the GL represent differentiated mature cells. The cells in the cartoon with different colors correspond to the stages of neuronal development in the bottom panel.

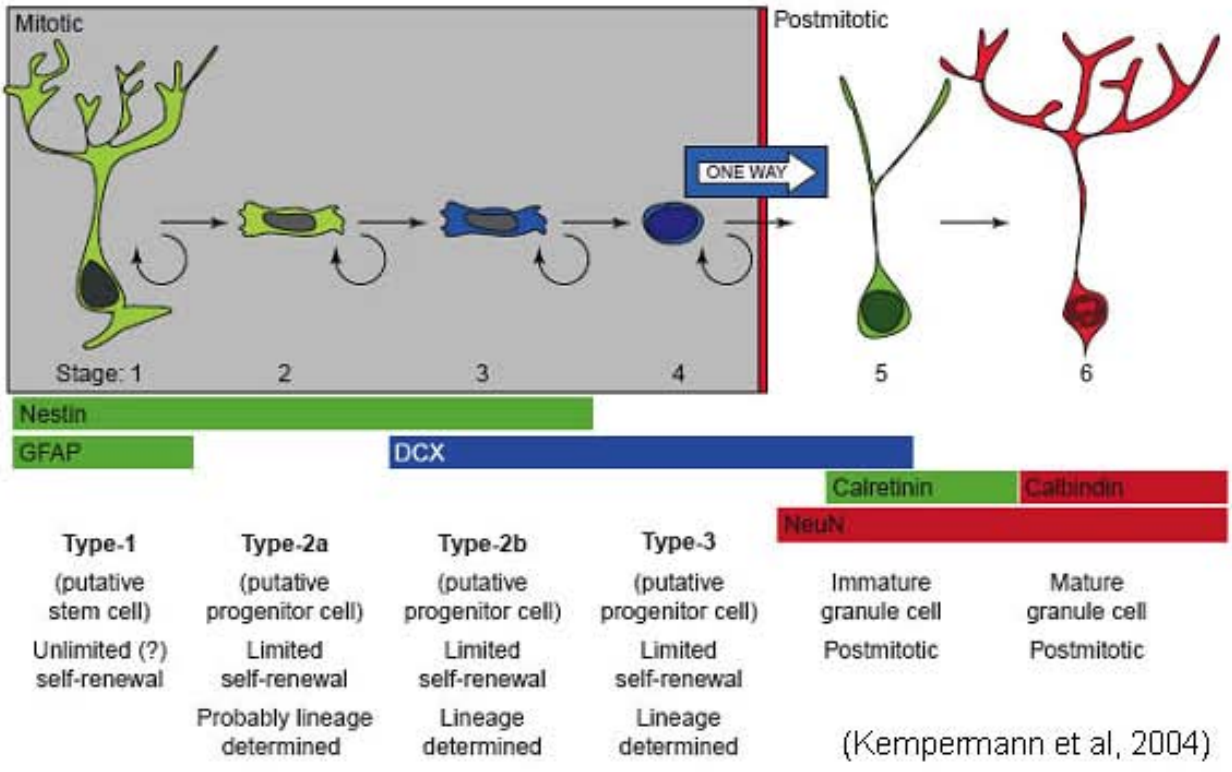

Figure I-3. Sequence of cell types in adult hippocampal neurogenesis. Six stages of neuronal development in the adult hippocampus are proposed on the basis of morphology, proliferative ability, and expression of markers. Each marker represents, nestin: neural stem cells, GFAP (Glial fibrillary acidic protein): astrocytes, DCX (Doublecortin): neuroblasts / migrating neurons, NeuN (Neuronal nuclei): mature neurons, calretinin: differentiating granule cells, calbindin: mature granule cells

Table I-1. Extrinsic factors regulating adult neurogenesis in the subventricular zone (SVZ) and subgranular layer (SGL)

Factors	SGL			SVZ		
	Cell proliferation	# of *de novo* neurons	Neuronal differentiation	Cell proliferation	# of *de novo* neurons	Neuronal differentiation
Hormone						
Corticosterone	−					
Estrogen	+					
Prolactin				+	+	
Neurotransmitters						
Dopamine	−			−		
Serotonin	+			+		
Acetylcholine	−					
Glutamate	−					
Norepinephrine	+					
Nitric oxide	−					
Growth or trophic factors						
FGF-2	0	0	0	+	+	+
EGF	0	−	−	+	−	−
IGF-1	+	+	+			
TGF-α				+		
BDNF				+	+	0
CNTF	+	+	+	+		
VEGF	+	+		+		
Morphogenic factors						
Shh	+	+	0			
BMP				−	−	−
Noggin				+	+	+
Behavior						
Enriched environment	+/0	+/0	+/0	0	0	0
Physical activity	+	+				
Learning	0	+				
Drugs						
Antidepressants	+					
Opiates	−					
Methamphetamine	−					
Pathological stimulation						
Ischemia	+/−	+	+	+		+
Seizures	+/−	+/0	+/0	+		
TBI	+	+				
Inflammation	−	−	−	+/−		

+: increase, −: decrease, 0: no change. Blank indicates not determined. This table was created on the basis of review articles [43,44].

CHAPTER II

MEMBERS OF THE NuRD CHROMATIN REMODELING COMPLEX INTERACT WITH AUF1 IN DEVELOPING CORTICAL NEURONS

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Running title: Chromatin Remodelers in Developing Cortical Neurons

ABSTRACT

Chromatin remodeling plays an important role in coordinating gene expression during cortical development, however the identity of molecular complexes present in differentiating cortical neurons that mediate the process remains poorly understood. The A+U-rich element-binding factor 1 (AUF1) is a known regulator of mRNA stability and also acts as a transcription factor upon binding to AT-rich DNA elements. Here we show that AUF1 is specifically expressed in subsets of proliferating neural precursors and differentiating post-mitotic neurons of the developing cerebral cortex. Moreover, AUF1 is co-expressed with Histone Deacetylase 1 (HDAC1) and Metastasis Associated Protein 2 (MTA2), members of the Nucleosome Remodeling and Histone Deacetylase (NuRD) complex. AUF1 specifically and simultaneously binds to HDAC1, MTA2 and AT-rich DNA element, its gene regulatory function is modulated by the extent of histone acetylation and in animals lacking AUF1, the composition of the complex is modified. These results suggest that AUF1 is involved in integrating genetic and epigenetic signals during cortical development through recruiting HDAC1 and MTA2 to AT-rich DNA elements.

KEYWORDS: cerebral cortex, epigenetics, gene expression, histone acetylation, neuronal differentiation, progenitors

INTRODUCTION

Normal cortical development is influenced by both genetic and epigenetic factors resulting in a precisely coordinated expression of genes in differentiating neurons. It has been shown that chromatin remodeling can play a significant role in corticogenesis (Berube et al. 2005), however, the full identity of molecules required for the developmental process is currently not known (Hsieh and Gage 2005). During our search for DNA binding proteins that are specifically expressed in the developing brain we found AUF1 (Dobi et al. 2006). Although we isolated AUF1 (also called hnRNPD (Dempsey et al. 1998)) based on its ability to bind AT-rich dsDNA, AUF1 was originally identified as an AU-rich element (ARE) binding protein (Brewer 2002; Lu et al. 2006; Zhang et al. 1993). Alternative splicing of exon2 and exon7 of the AUF1 primary transcript results in four isoforms, p37 (A), p40 (B), p42 (C), and p45 (D) (Brewer 2002; Laroia and Schneider 2002; Lu *et al.*, 2006; Wagner et al. 1998; Wilson et al. 1999; Zhang et al. 1993). AUF1 proteins are found both in the cytoplasm and also in the nucleus depending on the cell and tissue type (Blaxall et al. 2000; Sarkar et al. 2003) and they can perform various molecular regulatory functions. The p37 isoform has been shown to regulate mRNA stability (Sarkar et al. 2003), we showed that it also binds AT-rich dsDNA with high specificity and acts as a transcriptional regulator (Dobi et al. 2006). In addition, studies using various cell culture and *in vitro* models showed that the p40 isoform interacts with the TATA binding protein and regulates gene expression (Tolnay et al. 2000). The p42 isoform binds single-stranded telomeric C-strand, interacts with telomerase and is involved in regulating telomeric length (Enokizono et al. 2005; Eversole and Maizels 2000).

Our initial study showed high levels of AUF1 expression in the embryonic cortex, suggesting that AUF1 proteins may be involved in regulating corticogenesis (Dobi et al. 2006). As a first step toward understanding the molecular function of AUF1 in the developmental process, we set out to determine the phenotype of AUF1+ cells in the embryonic rat brain.

Because we found that the spatial and temporal pattern of AUF1 expression (Dobi et al. 2006) and HDAC1 and MTA2, members of the NuRD chromatin remodeling complex (Szemes et al. 2006), are remarkably similar in the developing rat brain, we designed experiments to determine if AUF1, HDAC1 and MTA2 are co-expressed and interact in developing cortical neurons. Lastly, we set out to determine the functional significance of the detected interactions by analyzing the effect of altered histone acetylation on the regulatory function of AUF1 using a primary embryonic cell culture model. Our results suggest that AUF1 is involved in coordinating gene expression of proliferating neural precursors/progenitors and post-mitotic neurons of the cerebral cortex through recruiting chromatin-remodeling molecules to AT-rich DNA elements.

MATERIALS AND METHODS

Animals

Timed-pregnant Sprague-Dawley rats (Zivic Laboratories Inc., Pittsburgh, PA) were used in these studies. All animals were treated in accordance with institutional and National Institutes of Health guidelines for the Care and Use of Laboratory Animals. Animal protocols were approved by the Institutional Animal Care and Use Committee at Uniformed Services University of the Health Sciences (USUHS).

Tissue preparation

Following Ketamine/Xylazine anesthesia, rat embryos were obtained from timed pregnancies, and their brains were removed and fixed by immersion in 4% paraformaldehyde (PF). Fixed brains were cryo-protected by immersing them in cold 15% and 30% sucrose solution in PBS. Frozen brains were cut coronally in 20 μm sections with a cryostat (Cryocut 1800, Leica, Bannockburn, IL). Sections were mounted on Colorfrost plus slides (Fisher Scientific, Suwanee, GA) and kept at -80 °C until use.

Flow Cytometric Analysis

Fluorescence-activated cell sorting (FACS) analysis was performed as described previously (Maric et al. 2003). Briefly, telencephalic tissues from E13 and E19 rat embryos were dissociated into single cell suspensions and cells were immunophenotyped using cell surface markers, tetanus toxin fragment C and A2B5. The cells were then fixed in 4% PF and subsequently labeled using anti-proliferating cell nuclear antigen (PCNA) and anti-AUF1 antibodies. The cells were enumerated and their immunoreactions quantified using the

FACSVantage SE flow cytometer and Cell Quest Acquisition and Analysis software (Becton Dickinson, Mountain View, CA), as previously described (Maric et al. 2003).

Immunohistochemistry

Immunohistochemistry was performed as described earlier (Szemes et al. 2006) using the following primary antibodies: AUF1 (1:500) (Lu and Schneider 2004); Phospho-Histone H3, (1:100, Cell Signaling Technology, Danvers, MA); nestin (1:1000, Chemicon, Temecular, CA); TuJ1 (1:1000, Babco, Berkeley, CA); HDAC1 (1:50, Upstate, Charlottesville, VA), MTA2 (1:200, Abcam, Cambridge, MA). For double immunohistochemistry, Zenon Rabbit IgG Labeling Kit was used according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Primary antibodies were visualized with the following secondary antibodies: Alexa Fluor 555 goat anti-mouse IgG (1:5000) and Alexa Fluor 488 goat anti-rabbit IgG (1:5000) (both from Invitrogen). Nuclei were counterstained using Hoechst 33342 (Molecular Probes, 1ug/ml applied for 2 minutes). Sections were analyzed in an Olympus IX71 microscope equipped with a Spot camera (Diagnostic Instruments, Sterling Heights, MI, USA). In order to assess the extent of co-expression, images were captured using a Zeiss Pascal Laser Scanning Confocal Microscope. The parameters of image acquisition (including laser power, detector gain, offset, etc.) were preset to maximize signal and minimize background and were kept constant (Gyorgy et al. 2008). We assessed the extent of AUF1 and MTA2 or AUF1 and HDAC1 co-expressions by counting red and green pixels of TIFF images using a Mathematica 5.2 script (Wolfram Research Inc. software, Urbana, IL; script written by Dr. J. Czege, Biomedical Instrumentation Center, USUHS). The script is freely available at http://bic.usuhs.mil/agoston/quantification.html. The script counts every pixel that has either a green and/or red value above the background threshold. The number of red, green and yellow (green + red) pixels, indicating co-expression, were counted and expressed as the ratio of pixels over the total numbers of colored pixels above the set threshold. Identical cortical regions of four independent double staining experiments were analyzed.

Western blot

Embryonic and neonatal rat whole brains were micro-dissected under a stereomicroscope and tissues were snap-frozen in liquid nitrogen and stored at -80^{o}C until used. The isolation of nuclear and soluble proteins, electrophoresis and transfer were described earlier (Dobi et al. 2006; Szemes et al. 2006). Twenty micrograms of proteins were loaded and separated as described. Transferred proteins were probed with AUF1 antibody as described (Dobi et al. 2006) or commercial AUF1 antibody (Upstate Biotechnology, Waltham, MA) both diluted at 1:1000. Immunoreactive bands were visualized using the SuperSignal ECL detection system (Pierce, Rockford, IL) and quantified using a Fuji LAS-1000 CCD Camera and the Fuji Image Gauge software (Fuji Photo Film USA, Valhalla, NY).

Co-immunoprecipitation assay

Nuclear and cytoplasmic proteins were prepared as described (Dobi et al. 2006; Szemes et al. 2006). Proteins were dialyzed against buffer D (20% glycerol, 20 mM HEPES (pH=7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM AEBSF) for 2 hours, changing the buffer after 50 minutes, at 4^{o}C, in the 3500 MWCO Slyde-A-Lyzer Mini Dialysis Unit (Pierce, Rockford, IL). For pre-clearing, 50 µg of nuclear extracts were incubated with 50 µl of the Protein A Sepharose bead slurry in 400 µl total volume under constant, slow rotation for 1 hour at 4 °C, then sedimented by centrifugation for 10 minutes at 10,000 g, also at 4^{o}C. The pre-cleared nuclear extracts were then immunoprecipitated with 2 µg of anti-AUF1 antibody or with 2 µg of control antibody (EGFR, Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 2 hours. Immunocomplexes were captured by adding 100 µl of the Protein A Sepharose bead slurry. After one additional hour of rotation at 4 °C the bound fraction was separated by pulse centrifugation. The beads were washed three times with 1 ml of ice-cold washing buffer (1x PBS

containing 0.1% Igepal). After the last wash, beads were re-suspended in 2x loading buffer containing 0.1 M DTT and LDS (lithium dodecyl sulphate) sample buffer (Invitrogen, Carlsbad, CA) denatured at 80^{o}C for 10 minutes, centrifuged at 10,000 g for 5 minutes at room temperature, and processed for Western analysis as described above.

DAPSTER assay

The DNA affinity preincubation specificity test of recognition (DAPSTER) assay was performed as described earlier (Kumar and Bernstein 2001) and optimized for the developing brain (Dobi et al. 2006). Nuclear proteins were pre-cleared with a streptavidin agarose bead slurry (ImmunoPure Immobilized Streptavidin, Pierce) without DNA. One hundred and fifty µg of the pre-cleared nuclear extracts were preincubated with dI/dC (6 µg/ml) for 10 minutes on ice followed by the addition of either 600 pmol of specific competitor ds rAT (5'rAT annealed with 3'rAT, for sequence information see **Table II-1.**) or 600 pmol of control competitor dsDNA (5' rAT_{Mut} annealed with 3' rAT_{Mut}) or kinase buffer only. Biotinylated AT-rich DNA probes (5'Bio-rAT) were annealed and 150 pmol of the dsBio-rAT was coupled to streptavidin beads, then washed with buffer Z (25 mM HEPES pH 7.9, 20% glycerol, 0.1% Igepal, 0.1 M KCl, 12.5 mM MgCl2, 1 mM DTT, 0.1 µM ZnCl2). Samples and beads containing the various immobilized DNA probes were mixed and incubated for an additional 2 hours at 4^{o}C. Bound fractions were then separated by pulse centrifugation and beads were washed three times with 1 ml of ice-cold buffer Z each. Beads were re-suspended in 2x LDS loading buffer, proteins denatured by boiling and samples were analyzed by Western blot as described above.

Chromatin immunoprecipitation (ChIP)

The assay was performed using the EZ-ChIP Kit from Upstate (Charlottesville, VA, USA) according to the manufacturer's instructions, and optimized to neonatal rodent brain tissue. All

buffers used were supplemented with protease inhibitors. Briefly, snap-frozen whole brain tissue pieces from P2 wild type and AUF1 mutant mice were transferred into 5 ml 1xPBS containing 1% formaldehyde and rotated for 15 minutes on room temperature. Cross-linking reactions were stopped by the addition of 500ul 10x Glycine Buffer, then washed twice with 1x PBS, and finally the crosslinked tissue was lysed with Lysis Buffer. Cells were disrupted by sonication on ice (six 10-second long bursts at tune 4, setting #6, each run separated by 60-second cooling breaks). After centrifugation, supernatants were aliquoted, then diluted with Dilution Buffer. Next, the samples were precleared in the presence of DNA and bovine serum albumin blocked protein A-coupled agarose beads for 60 minutes on 4 ^{o}C. The precleared supernatants were then incubated overnight at 4 °C with five µg of one of the following antibodies: AUF1 (Upstate, Charlottesville, VA), HDAC1 (Upstate, Charlottesville, VA), MTA2 (Abcam, Cambridge, MA), SATB2 (Szemes et al. 2006) or normal rabbit serum as a negative control. One aliquot was not immunoprecipitated and was later used as the input control sample. Then 60 µl of DNA- and bovine serum albumin-blocked, protein A-coupled agarose beads were added to each sample. The samples were then incubated under constant rotation overnight at 4 °C. The precipitated samples were washed once with 1 ml of Low Salt Buffer, once with High Salt Buffer, once with the LiCl Buffer, and twice with room temperature 1x TE buffer. After the final wash, the precipitated protein-DNA complexes were eluted by the addition of Elution Buffer containing 0.5% SDS and 0.1 M $NaHCO_3$, and after incubating for 30 minutes on room temperature, the supernatants were supplemented with NaCl to and end concentration of 0.3 M, and incubated overnight at 65 °C to reverse the crosslinking. Proteins and RNA were removed by the addition of ProteinaseK and RnaseH. The precipitated genomic DNA was purified using the QiaQuick PCR purification kit (Qiagen, Valencia, CA). Three ul of the purified DNA was used as a template in the first PCR; for the input sample, the DNA was diluted 100 times.

For the rat samples, the rENK2700-703-LUC plasmid containing the rat AT-rich DNA element was used as a positive control. Here, the first PCR was carried out using 25 cycles, with primers specific to the rAT region: 5'tAT-O and 3'rAT-O (**Table II-2.**) After the first PCR, amplicons were diluted 100 fold and used as templates in the second (nested) PCR reaction using 25 cycles with primers 5'rAT-N and 3'rAT-N. In the negative control, no DNA template was used, but an equal amount of PCR-grade water was instead added to the mix. The PCR products were separated on 2.5% agarose gel along with a 100 bp molecular weight ladder. Bands were visualised with ethidium bromide.

For the mouse samples, primers were designed to allow quantification of the products. Accordingly, all oligonucleotides were 24-mers, with a GC content of 50% (± 4) and a Tm of 60.0 °C (± 2.0), keeping the product size between 250 and 700bp (Strahl-Bolsinger et al. 1997). The first PCR was carried out using 25 cycles, with primers specific to the mouse AT region: 5'mAT-O and 3'mAT-O (**Table II-2.**). After the first PCR, amplicons were diluted 10 fold and used as templates in the second (nested) PCR using 25 cycles with primers 5'mAT-N and 3'mAT-N. GAPDH was used as a negative control gene, and was amplified with 5'GAPDH and 3'GAPDH primers from samples precipitated with SATB2, AUF1, MTA2 antibodies. The PCR products were separated on 1.5% agarose gel along with a 100 bp molecular weight ladder, and bands were visualised with SYBR-Green. The bands were visualized on a FUJI 3000 LAS intelligent dark box equipped with a CCD camera, and the band densities quantified using the ImageGouge software.

Cell cultures and transfection

Mouse embryonic fibroblast (MEF) cells were obtained from AUF1 mutant and wild type animals (Lu et al. 2006). MEF-AUF1$^{-/-}$ and MEF-AUF1$^{+/+}$ cells were cultured in DMEM in the presence of 2 mM L-glutamine and 10% heat-inactivated fetal calf serum. The AT-rich DNA element was inserted in the plasmid pTK-Renila (Promega, Madison, WI) resulting the reporter

plasmid pTK-rAT-Ren in which the baseline activity of Renila luciferase is modified by AUF1 proteins (Dobi et al. 2006; Szemes et al. 2006). In the control reporter plasmid TK-rAT$_{mut}$-Ren the AT-rich DNA element was mutated so the resulting sequence failed to bind AUF1 (Dobi et al. 2006). Cells were transfected using the PEI transfection system as previously described (Szemes et al. 2006) with 1.5 µg of the reporter plasmids (TK-rAT-Ren or TK-rAT$_{mut}$–Ren) and 0.1 µg of RSV-Firefly Luciferase (Promega, Madison, WI) enabling normalization for transfection efficiency. Forty-eight hours after transfection, some of the cultures were treated with 1 mg/ml of trichostatin A (TSA), a blocker of histone deacetylases dissolved in ethanol. Control cultures received equal volume of vehicle only**.** After 18 hrs of treatment, cultures were harvested and Firefly and Renila luciferase activities were measured using the Dual-Luciferase Assay System according to the manufacturer's instructions (Promega, Madison, WI). Relative light units (RLU) of Renila Firefly luciferase activities were normalized by Firefly RLUs representing the activities of the control plasmid (RSV-Firefly Luciferase), as recommended by the manufacturer (Promega, Madison, WI).

RESULTS

AUF1 expression is developmentally regulated in the rat brain

We determined the relative abundance of AUF1 proteins in tissue extracts prepared from E14, E15, E16, E18, P2, and P28 brains by Western blotting using pan-specific AUF1 antibodies that recognize all isoforms in an unbiased fashion (Lu and Schneider 2004). Semi-quantitative assessment of band intensities showed that the level of expression of AUF1 proteins did not substantially change from E14 to P2 (Figure II-1). The commercial antibody also reacted with a ~35-kDa protein marked with star (see Supplementary Figure II-1). During development, AUF1 protein levels steadily declined and after birth (P2) the abundance of AUF1 proteins was ~ 30% of the E14 brain. Although AUF1 proteins can be detected in the P28 brain, their abundance was substantially lower than in the developing brain. Western analysis also showed that all AUF1 isoforms were expressed at all ages tested and that the ratio between the different isoforms was practically identical throughout development (E14 to P2). Of the various isoforms, p40/42 were the most abundant. Although the abundance of AUF1 proteins in the adult brain was very low as compared to the developing brain, it appears that the ratio between the isoforms in the adult brain was similar to that detected in the developing brain.

AUF1 is expressed by proliferating precursors/progenitors and postmitotic differentiating neurons in the cerebral cortex

The high level of AUF1 expression in the E14 brain suggested that AUF1 was expressed in proliferating progenitor cells that were the most abundant cell type at this developmental stage (Ohnuma and Harris, 2003). We first carried out quantitative FACS analysis using a previously established immunophenotyping paradigm (Maric et al. 2003) to test the proliferative status of AUF1+ cells. We isolated cells from E13 telencephalon at the beginning of cortical neurogenesis, when the majority of neural precursor cells were proliferating and from E19

cortex at the end of cortical neurogenesis, when most of the neuronal cells were post-mitotic. At both developmental stages, we quantified the numbers of AUF1+, PCNA+ and double-positive cells. We found that at E13 approximately 70% of the total neural precursor population was positive for both PCNA and AUF1 indicating that the large majority of AUF1 expressing cells were proliferating (Figure II-2A, C and Supplementary Figure II-2A, B). In contrast, only ~3% of the total neural precursor population was PCNA+/AUF1+ at E19. The majority (~60%) of these cells were PCNA-/AUF1+, indicating that AUF1 was widely expressed in the post-mitotic/differentiating compartment of the cortical neuronal precursor cell population at this stage of cortical development (Figure II-2B, C).

We performed immunohistochemical analysis to identify the developmental phenotype of AUF1+ cells in the embryonic and early postnatal cortex. Consistent with our FACS data, during early brain development (E14) most of AUF1+ cells were located in the ventricular zone (VZ) and in the subventricular zone (SVZ), the site of proliferating precursors/progenitors (Figure II-3A). Double immunohistochemistry using AUF1 antibody and an antibody raised against Phospho-Histone H3 (PhH3), a marker of proliferating cells in the M phase of cell cycle (Brenner et al. 2003; Rayzman and Sentry 2006) showed that virtually all PhH3+ cells in the ventricular zone also expressed AUF1 (Figure II-3B, C). Consistent with the data obtained from FACS analysis, our double immunohistochemistry using AUF1 and nestin antibodies (Kim et al. 2003) showed that at E14 the majority of AUF1+ cells expressed nestin (Figure II-3, D-F) suggesting that during early brain development AUF1 was expressed by neural precursor/progenitor cells. In addition to the VZ and SVZ, we found AUF1+ cells located in the telencephalic wall (Figure II-3A, D), suggesting that AUF1 was also expressed in differentiating cortical neurons. Indeed, at E18 AUF1+ cells were located almost exclusively in the differentiating region of the cortex, especially in the cortical plate (CP) and in the marginal zone (MZ) (Figure II-3G). The majority of these AUF1+ cells also expressed TuJ1, a marker of postmitotic, differentiating neurons (Figure II-3H, I). In the P2 brain, the number of AUF1+ cells was substantially decreased (Figure II-3J).

AUF1+ cells were present in all layers, however, layer I contained the fewest, while layer II (MZ) contained relatively higher numbers of immunoreactive cells. Practically all AUF1+ cells were also positive for MAP2 (Figure II-3K, L), indicating that in the postnatal brain, AUF1-expressing cells were postmitotic differentiating neurons. In summary, our FACS and immunohistochemistry data suggest that AUF1 was expressed by proliferating neural precursors as well as by postmitotic, differentiating cortical neurons. We found that other brain regions were mostly devoid of AUF1+ cells with the exception of the pallidal SVZ, which contained some AUF1+ cells, as we also reported earlier (Dobi et al. 2006).

***AUF1 is co-expressed and interacts with HDAC1 and MTA2 in developing neurons of the cerebral cortex* in vitro *and* in vivo**

Because the spatial and temporal patterns of expression of HDAC1 and MTA2 in the developing cortex (Gyorgy et al. 2008) appeared to be very similar to the pattern of AUF1 expression shown above, we analyzed the pattern of co-expression by immunohistochemistry in E14, E18 and P2 brains. We found that in the developing cortex, AUF1 was co-expressed with both HDAC1 and MTA2 at all ages tested. In the E14 brain, semi-quantitative assessment showed that ~85% of AUF1+ cells expressed HDAC1 and ~ 95% of them expressed MTA2 (Figure II-4B, C). At E18, ~70% of AUF1+ cells in the cortical plate (CP) and in the marginal zone (MZ) co-expressed HDAC1 and ~80% of AUF1+ cells expressed MTA2. The extent of co-expression in the P2 cortex was similar to that of observed in the E18 cortex and semi-quantitative assessment of co-expression also indicated that ~80% of AUF1+ cells expressed HDAC1 and also MTA2. Interestingly, differentiating cells in other major brain regions such as the thalamus that lack AUF1 expression were also negative for HDAC1 and MTA2. Higher power confocal microscopy confirmed the nuclear co-expression of all three proteins at all developmental ages investigated (Supplementary Figure II-3).

As a first test toward understanding the potential functional significance of the co-expression of AUF1 with the selected chromatin remodeling molecules, we performed a co-immunoprecipitation assay using nuclear extracts prepared from E18 cortex. Following incubation with the AUF1 antibody, we analyzed the immunoprecipitated fractions for the presence of HDAC1 and MTA2 by Western blotting. The analysis showed that both HDAC1 and MTA2 interacted with AUF1 as indicated by the presence of ~80 kDa and ~ 60 kDa bands (Figure II-5A). We also detected an additional band at ~78 kDa, which may be a proteolytic product of MTA2, since the antibody should not cross-react with MTA1 according to the manufacturer (Abcam, Cambridge, MA). We did not detect either of the proteins when we replaced the AUF1 antibody with the control antibody suggesting that both HDAC1 and MTA2 specifically bind to AUF1 *in vitro.* In the reverse immunoprecipitation experiment, HDAC1 antibody also co-precipitated AUF1 as well as MTA2 (Supplementary Figure II-4).

We first identified and isolated AUF1 in the developing cortex based on its specific binding to AT-rich dsDNA elements (Dobi A *et al.*, 2006). Because AUF1 was co-expressed with, and binds to HDAC1 and MTA2, we hypothesized that the molecular function of AUF1 includes recruiting HDAC1 and MTA2 to AT-rich DNA regions. To test this hypothesis, we performed a DAPSTER, a microscale DNA affinity purification assay enabling the identification of proteins that specifically bind to the test DNA sequences (Kumar and Bernstein 2001). For the DAPSTER assay, we immobilized the same AT-rich dsDNA that we used to isolate AUF1 from the developing rodent cortex (Dobi et al*.* 2006) and performed the assay using nuclear extracts isolated from the P2 cortex that were pre-incubated with specific (rAT) or non-specific (rAT_{MUT}) oligonucleotides. As a positive control, we also performed this DNA affinity purification without competing DNA. Western analysis of the bound and free fractions showed that in addition to AUF1, both HDAC1 and MTA2 were present in the DNA-protein complex when no competitor or the non-specific competitor was present (Figure II-5B). Since neither HDAC1 nor MTA2 possess DNA binding domains (Yao and Yang 2003; Zhang et al. 1999) their presence in

the AT-rich dsDNA-binding complex was likely mediated through AUF1. The formation of the AT-rich dsDNA-protein complex was specific because preincubation of nuclear extracts with excess AT-rich dsDNA (rAT) used as a specific competitor almost completely abolished the binding of all proteins. Preincubation of the nuclear extracts with the non-specific / control competitor dsDNA (rAT_{MUT}) on the other hand did not significantly affect the formation of the complex.

We performed the ChIP assay to test whether the demonstrated *in vitro* interactions between AT-rich dsDNA and the three proteins also take place *in vivo*. For the ChIP assay, we used microdissected cortices from P2 rats because all three proteins were still expressed and the P2 cortex yielded sufficient cortical tissue required for the crosslinking studies (Dobi et al. 2006). Following paraformaldehyde crosslinking, we incubated the cortical samples with anti-AUF1, anti-HDAC1, anti-MTA2 antibodies or a negative control antibody. Following multiple washing steps, we isolated DNA from immunoprecipitated samples and used it as a template in PCR along with primers that flank the AT-rich dsDNA element (Dobi et al. 2006). Our results showed that the AT-rich DNA region could be amplified from the samples precipitated with AUF1, HDAC1 as well as MTA2 antibodies but not with the control antibody (Figure II-5C). These results demonstrated that AUF1, HDAC1 and MTA2 were all associated with the AT-rich DNA element *in vivo* and implicating AUF1 in recruiting both HDAC1 and MTA2 to AT-rich DNA regions.

Altering histone acetylation modulates the effect of AUF1 on gene expression

As a first step toward identifying the potential role of the detected interactions between AT-rich dsDNA, AUF1, HDAC1 and MTA2 in regulating gene expression, we used MEF-$AUF1^{-/-}$ and MEF-$AUF1^{+/+}$cells for our transient transfection analysis. We selected these cells for our initial studies because the cultures were composed of identical population of embryonic primary cells that differ only in the presence of AUF1, they can be transfected with high efficiency and they

endogenously express HDAC1 and MTA2. The presence of AUF1 decreased reporter gene expression by approximately 50% and TSA treatment of these cells caused a substantial increase in reporter gene activity (Figure II-6). Importantly, mutant cells lacking AUF1 (MEF-AUF1$^{-/-}$) showed no response to TSA treatment. TSA treatment had no effect on reporter gene activities when MEF-AUF1$^{+/+}$ and MEF-AUF1$^{-/-}$ cell cultures were transfected with the control reporter plasmid TK-rAT$_{mut}$–Ren. These results suggested that the molecular function of AUF1 depends on its specific binding to the AT-rich DNA site and involves directing the effect of histone acetylation or de-acetylation to these AT-rich DNA elements.

Absence of AUF1 alters the composition of the NuRD complex

To understand the molecular mechanism of AUF1 function *in vivo*, we analyzed the effect of lack of AUF1 on the composition of the NuRD complex in the developing brain. Using semi-quantitative ChIP assay, we compared the relative amounts of HDAC1 and MTA2 associated with the AT-rich DNA in the neonatal brains of AUF1 null mutant and wild type animals. The primers were designed and the conditions of the PCR were adjusted enabling semi-quantitative analysis (Strahl-Bolsinger et al. 1997).

We have found that in the absence of AUF1, the relative proportion of HDAC1 increased about two-fold (Figure II-7). This increase was specific to the AT-rich DNA region because there was no difference between the wild type and mutant animals when the GAPDH control region was amplified from samples precipitated with any of the antibodies used. Interestingly, the relative amount of MTA2 in the complex did not change significantly in the AUF1 mutant brain.

DISCUSSION

The goal of our present study was to gain an understanding about the role of AUF1 in the developing cortex. Based on our data presented above we propose that AUF1 proteins are involved in coordinating gene expression in proliferating neuronal precursors/progenitors and differentiating cortical neurons by recruiting chromatin remodeling molecules to AT-rich DNA elements. This is important because studies have shown that chromatin remodeling can play important roles at various stages of corticogenesis by coordinating the expression of multiple genes (Berube et al. 2005). At the molecular level, they provide permissive or restrictive chromatin environment for classical transcription factors during cellular differentiation, however, the identity of proteins directing their effect to specific genomic loci is little known (Hsieh and Gage 2005). AUF1 was recently also found to regulate DNA methyltransferase 1, an important determinant of genome-wide DNA-methylation (Torrisani et al. 2007).

We found that all AUF1 isoforms were expressed in the developing cortex and the ratio between the various isoforms did not change significantly between E14 and P2. AUF1 proteins were highly expressed in the E14 brain by proliferating cells. In addition to neural precursor/progenitor cells that were dividing by asymmetrical cell divisions (Ohnuma and Harris 2003). AUF1 may also be expressed in self-renewing neural stem cells that undergo limited cycles of symmetrical cell divisions. In the E14 brain, AUF1+ cells in the VZ express PH3, indicating that AUF1 was expressed during in the M phase of cell cycle. During M phase, inherited determinants of cell fate are localized to only one pole of the mother cell undergoing asymmetric cell division, thus only one of the daughter cells will inherit such a determinant (Bally-Cuif and Hammerschmidt 2003; Ohnuma and Harris 2003). Whether AUF1 falls into this category, *i.e.* it is inherited by only one daughter cell, needs to be addressed in future experiments. Our preliminary BrdU labeling studies indicated that AUF1 was also expressed in BrdU-positive cells (see Supplementary Figure II-5). Because chromatin remodeling during S

phase is thought to play a major role in regulating cellular differentiation (Ohnuma and Harris 2003) and because differentiating neurons change their fate according to their environments if they were in S (or G1) phase (McConnell, 1988), AUF1 could be involved in regulating neuronal differentiation through altering the access of transcription factors to their DNA binding sites.

Our double-immunohistochemical experiments showed an almost complete co-expression of AUF1 proteins with HDAC1 and MTA2 at a single cell level and ChIP assay demonstrated direct, simultaneous interaction between AUF1, HDAC1, MTA and the AT-rich dsDNA *in vivo*. Previous studies have also shown that developmental regulators such as BRG1 (Matsumoto et al. 2006) and REST-NRSF (Ballas and Mandel 2005; Lunyak and Rosenfeld 2005) regulate fate determination of neural stem cells and/or neural differentiation through their interaction with members of the mSin3A/B chromatin remodeling complex that contains HDAC1 (as well as HDAC2) (Ahringer 2000). Another chromatin remodeling complex that contains HDAC1 is the NuRD complex (Ahringer 2000; Bowen et al. 2004; Feng and Zhang 2003). The NuRD complex regulates chromatin structure through multiple mechanisms including histone deacetylation. Both HDAC1 and MTA2 are members of the complex and blocking histone deacetylase activity by TSA or valproic acid resulted in promoting a neuronal fate at the expense of a glial one (Hsieh and Gage 2004; Shen et al. 2005).

Our immunohistochemical studies have shown that AUF1 remains expressed in postmitotic differentiating cortical neurons and that AUF1 was also co-expressed in these cells with HDAC1 and MTA2. These findings implicate AUF1 in regulating gene expression at different stages of neuronal lineage progression through chromatin remodeling (Recillas-Targa and Razin 2001). Accumulation of acetylated histone molecules prevents chromatin condensation and the resulting open chromatin structure permits the binding of sequence-specific transcription factors to their cis-regulatory DNA elements, thus resulting in increased transcription of target genes (Alvarez et al. 2000). Conversely, histone deacetylation mediated by HDACs results in a closed chromatin structure, thus preventing the binding of transcription

factors to their DNA elements (Mal et al. 2001). In this study, we focused on the NuRD complex because it has been shown that the complex plays an important regulatory role in the differentiation of multiple cell types including neural cells (Feng and Zhang 2003). The complex regulates chromatin structure through multiple mechanisms that include histone deacetylation and nucleosome remodeling (Yao and Yang 2003). The complex is approximately two MDa in size, comprising at least seven polypeptides that include HDAC1 and MTA2. HDAC1 - along with HDAC2 – is also part of the Sin3a chromatin-remodeling complex (Zhang et al. 1999). Because MTA2 is not a part of the Sin3a complex (Ahringer 2000), the co-expression of HDAC1 and MTA2 in AUF1+ cortical neurons suggests that the chromatin-remodeling complex is NuRD rather than Sin3a. Since hypoacetylated histone amino-termini are generally correlated with transcriptional repression (Yao and Yang 2003), recruiting the NuRD complex to AT-rich DNA by AUF1 should result in repressor activity. Indeed, using a model system we have shown that AUF1 is important in directing the effect of altered histone de-acetylation, *i.e.* closed chromatin structure to AT-rich dsDNA resulting in decreased reporter gene activity.

AT-rich DNA elements appear frequently in the genome (Woynarowski 2004). AT-rich sequences can form three-dimensional binding sites for nuclear proteins that recognize three-dimensional structure rather than a short nucleotide sequence (Laemmli and Tjian 1996; Strick and Laemmli 1995; Strick et al. 2000). AUF1 thus joins the family of AT-rich DNA binding proteins that include nucleolin (Dickinson and Kohwi 1995), histone H1 (Pauli et al. 1989), HMGI/Y (Reeves 2000), SATB1 (Dickinson et al. 1992) and SATB2 (Britanova et al. 2005; Szemes et al. 2006). SATB1 is especially interesting because it interacts with and recruits members of the NuRD (and also CHRAC and ACF) chromatin remodeling complexes to AT-rich DNA sites and regulates nucleosome positioning over several kilobases (Yasui et al. 2002). Previous studies showed that these AT-rich DNA binding proteins, AUF1, SATB1 and SATB2, are expressed in a non-overlapping fashion during cortical development and they interact with both the nuclear matrix and with chromatin remodeling complexes (Britanova et al. 2005; Dobi

et al. 2006; Gyorgy et al. 2008; Szemes et al. 2006). These observations raise the intriguing possibility that there may be a development-specific modification of the chromatin structure in differentiating cortical neurons influenced by both genetic and epigenetic factors. This is important because despite the great progress made in identifying transcription factors and signaling molecules involved in regulating corticogenesis (Rakic 2006), we still lack a good understanding about the identity of molecules that integrate environmental signals and genetic programs believed to play a key role in coordinating gene expression (Jaenisch and Bird 2003). The surprising change in the relative abundance of HDAC1 in the NuRD complex of AUF1 mutant animals suggests that altered abundance of the key members of the complex may alter the expression levels of downstream genes. Our preliminary microarray data analyzing the transcriptome of AUF1 mutant brains indeed showed that the expression levels of genes associated with cell-adhesion and migration are altered in the mutant animals (data not shown). These molecular abnormalities may contribute to the apparent callosal dysgenesis of AUF1 mutant animals (see Supplementary Figure II-6).

Understanding how AUF1 fits into the regulatory network of molecules that control gene expression during cortical development is currently not known. The majority of the identified transcription regulators of cortical development are classical short DNA sequence specific binding proteins (Guillemot 2005; Kageyama et al. 1997; Sommer et al. 1996). Mutations of these genes result in specific developmental deficits. For example, in the absence of Dlx1/Dlx2, the “master” regulators of telencephalic development, the regionalization of the telencephalon is abnormal and there is a deficit in a subset of cortical interneurons (Cobos et al. 2005). Whether AUF1 acts up or downstream from Dlx and other classical regulators of cortical development is currently unknown. Because knockout animals, including Dlx1/2 double mutants, are available (Cobos et al. 2005), experiments can be designed to address this important issue. Additional studies will be also needed to identify which one(s) of the multiple molecular regulatory

functions of AUF1, regulating mRNA stability (Brewer 2002), gene transcription (Dobi et al. 2006) and chromatin remodeling (this paper) is/are required during corticogenesis.

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CHAPTER II Figures

Table II-1. Sequence information for oligonucleotides used in the DAPSTER assay

Name	*Sequence 5'-3'*
5'rAT	GAA CCA AAA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATA ATT AAC CAT
3'rAT	ATG GTT AAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TAT TTT TGG TTC
5'rAT$_{Mut}$	GAA CTG TTC TTA CTA TCG CCA TCA CCG TTA TTG CTC AAT TCG TAC TAG TTC ATG AAG TCT TTG G
3'rAT$_{mut}$	CCA AAG ACT TCA TGA ACT AGT ACG AAT TGA GCA ATA ACG GTG ATG GCG ATA GTA AGA ACA GTT C

Table II-2. Sequence information and physical characteristics of oligonucleotides used in the Chromatin Immunoprecipitaiton (ChIP) assay

Name	*Sequence (5'-3')*	*Product size*	*GC%*	*Length (nt)*	*Tm (C^o)*
5'mAT-O	ACT CGT GTT AGC CTC TGA GCT AGT	550	50	24	60.15
3'mAT-O	GTG ACA CTA GCA GAC CAG ATT CCT	550	50	24	61.08
5'mAT-N	GCC TCT GAG CTA GTA TGT GTC ATC	274	50	24	59.46
3'mAT-N	CCA TCA CCT TCT CCT TTC TAG GTC	274	50	24	61.64
5'rAT-O	TTT AGC TCA GTG GTA GAG CGC	257	52	21	56
3'rAT-O	GTT TCT GCC CTT TCC AAC TGC	257	52	21	56
5'rAT-N	CCC TGG GTT CGG TCC CCA GC	131	75	20	58
3'rAT-N	GTT CCC AGA CCT GTC CAG TTC	131	57	21	53
5'GAPDH	ATC ACT GCC ACC CAG AAG ACT G	623	67	22	60
3'GAPDH	CCC TGT TGC TGT AGC CGT ATT C	623	65	22	61

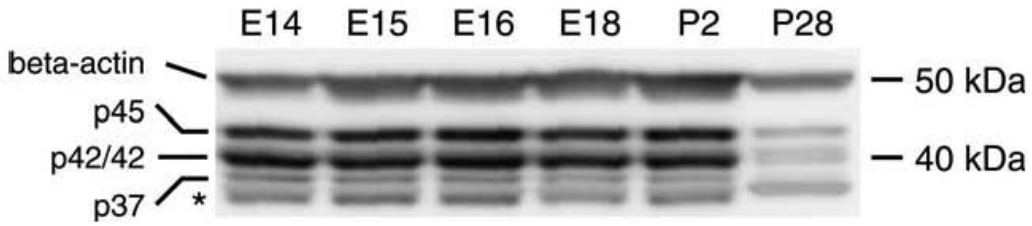
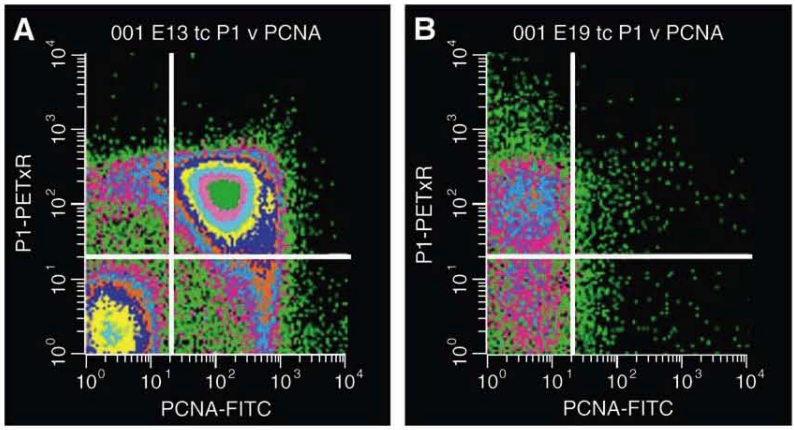


Figure II-1. Expression of AUF1 proteins in the developing and adult rat brain. Representative Western blot analysis showing the relative abundance of the various isoforms at the selected ages. Star indicates the nonspecific band recognized by the anti-AUF1 antibody.



C

PCNA vs. AUF1

	E13 (%)	E19 (%)
UL	3.72	60.49
UR	71.10	3.11
LL	19.59	33.25
LR	5.59	3.16

Figure II-2. The proliferative state of AUF1-expressing cells in the rat brain. The bivariate dot density plot derived from FACS analysis shows the expression(s) of PCNA and AUF1 in the mostly proliferating neural precursor/progenitor population isolated from the E13 rat telencephalon (A) and their predominantly postmitotic/differentiating counterparts isolated from E19 rat cortex (B). Cell population in the upper right quadrant represents the contribution of proliferating AUF1+ cells to the total cell population at the 2 developmental ages. The developmental changes in the percentage distribution among the various cell populations are tabulated (C). Upper-left (UL) represents $AUF1^{+}/PCNA^{-}$, upper-right (UR) represents $AUF1^{+}/PCNA^{+}$, lower-left (LL) represents $AUF1^{-}/PCNA^{-}$, and lower-right (LR) represents $AUF1^{-}/PCNA^{+}$ fraction, respectively.

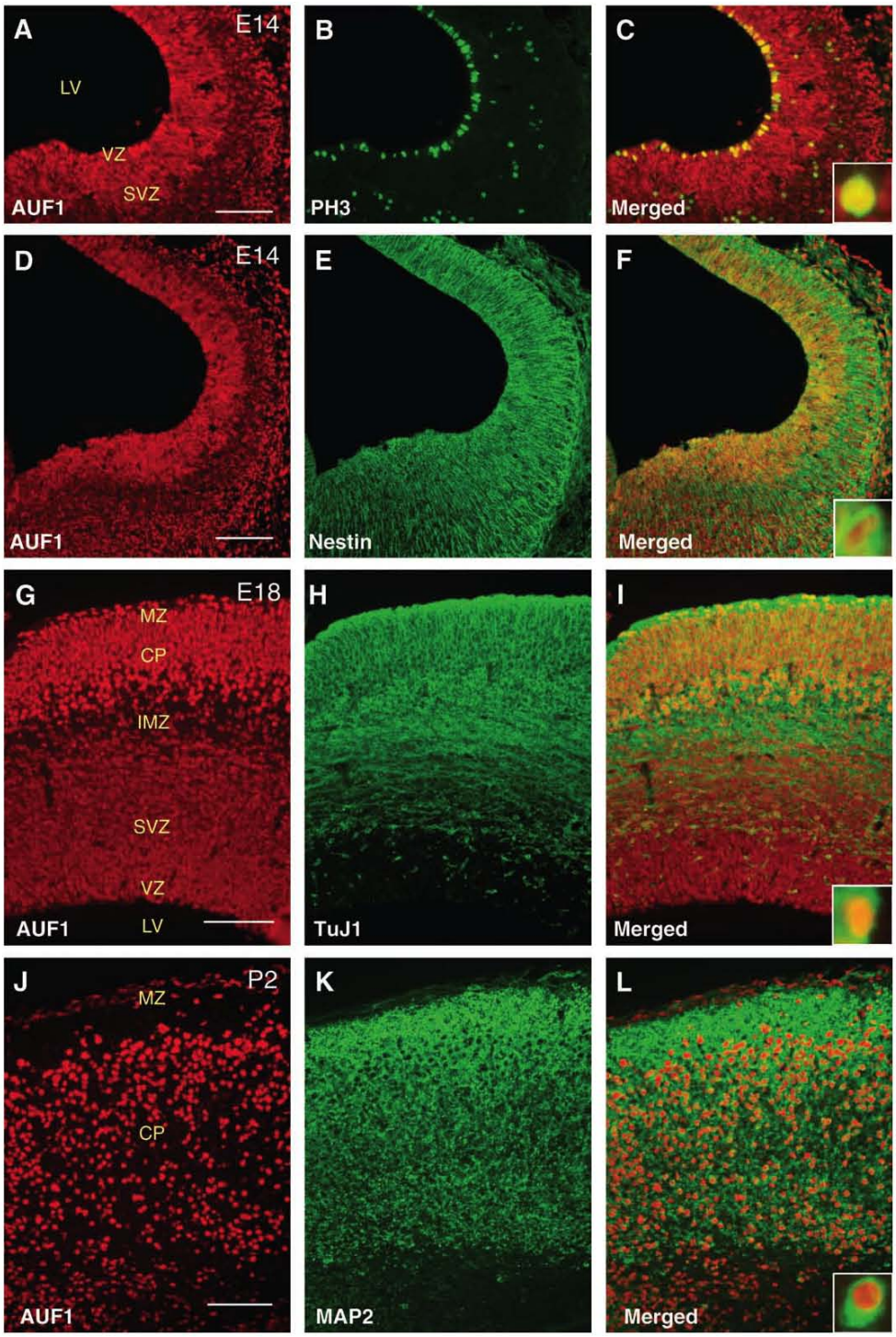


Figure II-3. The developmental phenotype of AUF1-expressing cells in the rat brain. Double immunohistochemistry (A-C) of E14 rat coronal sections show the location of proliferating Phospho-Histone H3+/AUF1+ cells. At this age, most nestin+ cells that are located in the VZ and SVZ express AUF1 (D-F). At E18, most AUF1+ cells that are located in the MZ and CP of the developing cortex express TuJ1 (*G-I*). After birth, the number of AUF1+ cells decreases substantially and the remaining AUF1+ cells express MAP2 (*J-L*) at P2. LV = lateral ventricle; VZ = ventricular zone; SVZ = subventricular zone; MZ = marginal zone; CP = cortical plate; IMZ = intermediate zone, Scale bars in A-F = 200 μm and in G-L = 100 μm.

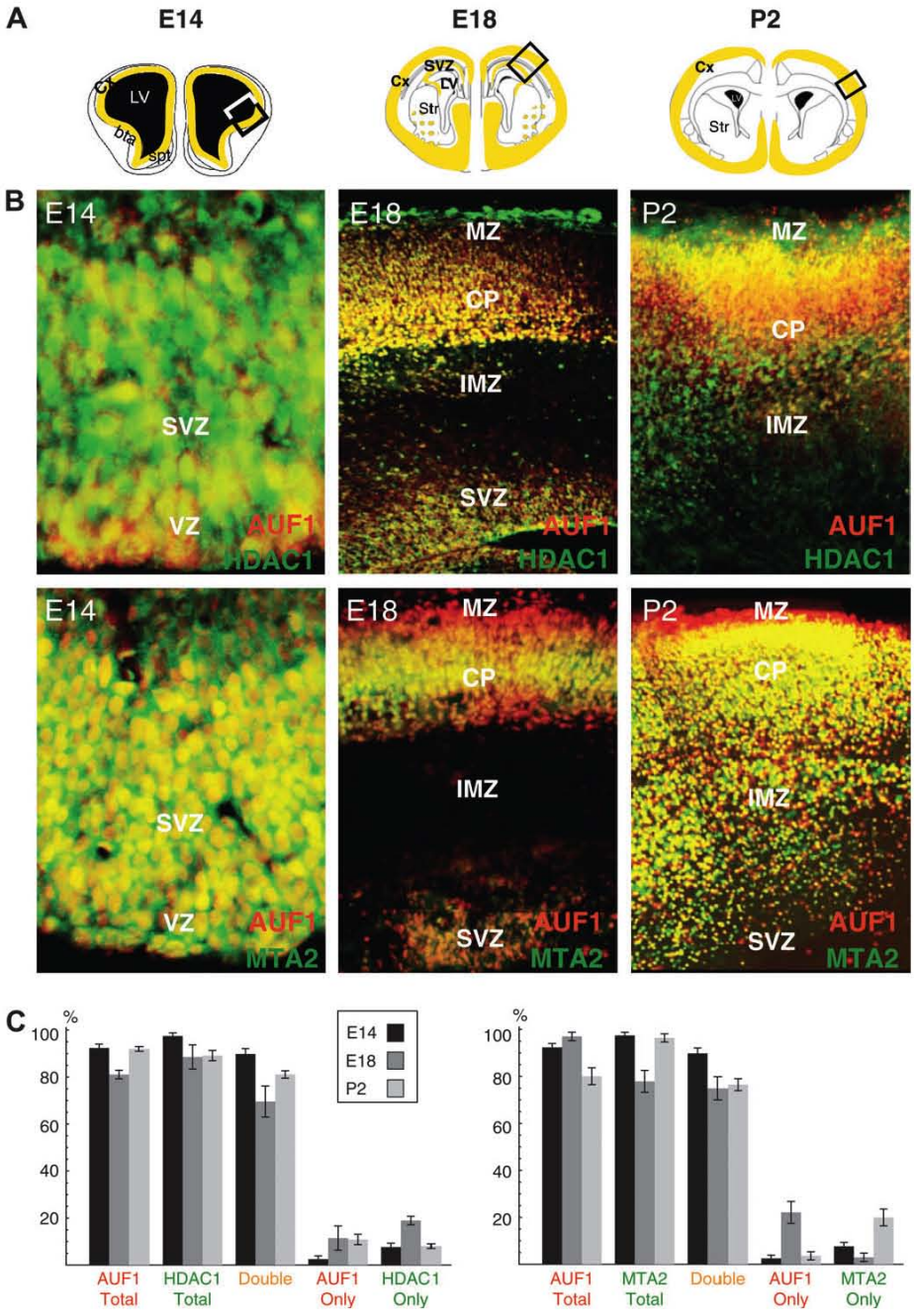


Figure II-4. Pattern of coexpression of AUF1, HDAC1, and MTA2 in the developing rat brain. Anatomical maps of coronal sections of the developing rat brain at ages E14, E18, and P2 (*A*) summarizing the patterns of co-expression (yellow colored areas). Left hemispheres: AUF1/HDAC1; right hemispheres: AUF1/MTA2. Abbreviations: Bta = Basal telencephalon; Spt = septal neuroepithelium; Lv = lateral ventricle; Str = striatum; Cx = cortex. Representative double-immunohistochemical images showing the pattern of AUF1, HDAC1, and MTA2 expression in the developing rat brain (*B*). Coronal sections from E14, E18 and P2 rat brains were immunostained using a combination of AUF1 and HDAC1 (upper row) and AUF1 and MTA2 (lower row) antibodies. Abbreviations: Cx: cortex; LV: lateral ventricle; IMZ: intermediate zone; Scale bar = 1mm. Graphs showing the extent of coexpression (AUF1/HDAC1 and AUF1/MTA2) from E14 to P2 in the developing rat cortex. The bar graphs show the ratio of red (AUF1), green (HDAC1 or MTA2) and yellow (double-positive) areas expressed as percentage of the total area scanned (*C*). Vertical axis = percentage of total color pixels. N=4.

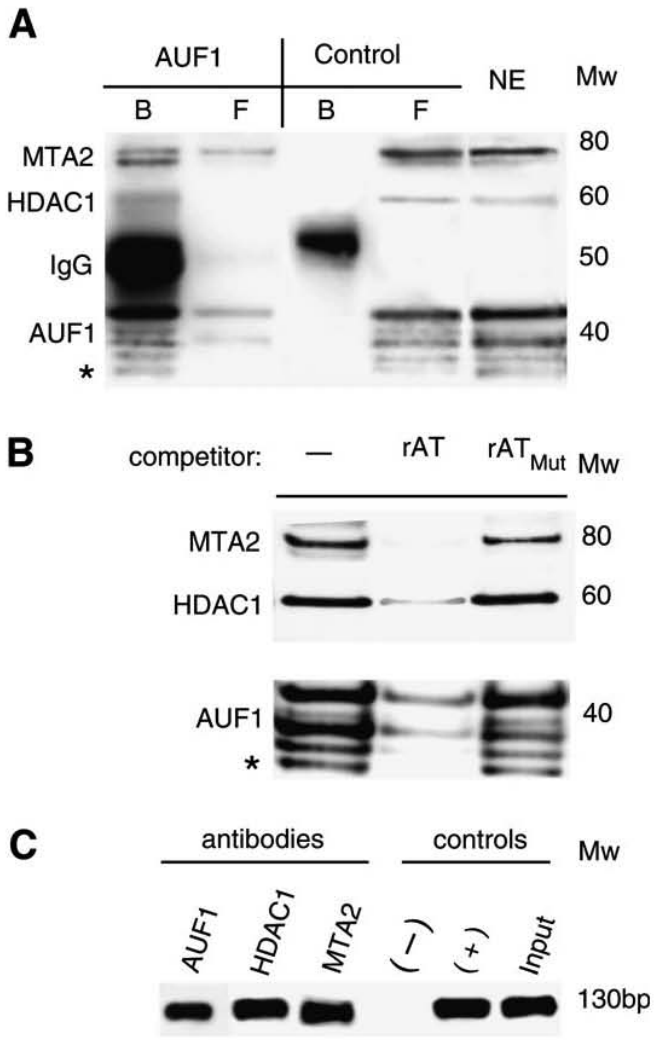


Figure II-5. *In vitro* and *in vivo* interactions of AUF1 with HDAC1, MTA2, and AT-rich DNA. Nuclear extracts (NE) were prepared from the E18 rat cortex and immunoprecipitated with either AUF1 or control antibody. Following immunoprecipitation, bound (B) and free (F) fractions were analyzed by Western blots using specific antibodies. The identities of AUF1-, MTA2-, and HDAC1-immunoreactive bands are marked on the left. Molecular weight (MW) is shown in kDa on the right side. Star indicates a non-specific band (*A*). The DAPSTER assay was performed using nuclear extracts prepared from the E18 rat cortex. DNA affinity assay was either carried out in the absence of competitors (-), or in the presence of the specific competitor rAT or control rAT_{MUT} dsDNA. The presence of the AUF1, HDAC1 and MTA2 in the DNA-protein complex was determined by Western blot using specific antibodies. The identities of AUF1-, MTA2- and HDAC1-immunoreactive bands are marked on the left (*B*). A ~150-bp DNA fragment containing the rAT DNA region was amplified from samples that were immunoprecipitated with AUF1, HDAC1 or MTA2 antibodies and PCR amplicons were separated on agarose gels. No DNA was amplified from samples that were immunoprecipitated using the preimmune serum to AUF1 or a control antibody (EGFR). The ChIP assay was performed on postnatal (P2) rat cortical tissue. Precipitated DNA fragments were amplified by PCR, then the product of the nested (2nd) PCR was subjected to agarose gel electrophoresis and DNA fragments were visualized by ethydium bromide staining under UV light. Input sample indicates the nonimmunoprecipitated positive control; plasmid DNA containing the rAT region used as template for PCR amplification was used as another positive control (+). For the negative control (-) we used the EGFR-precipitated sample (*C*).

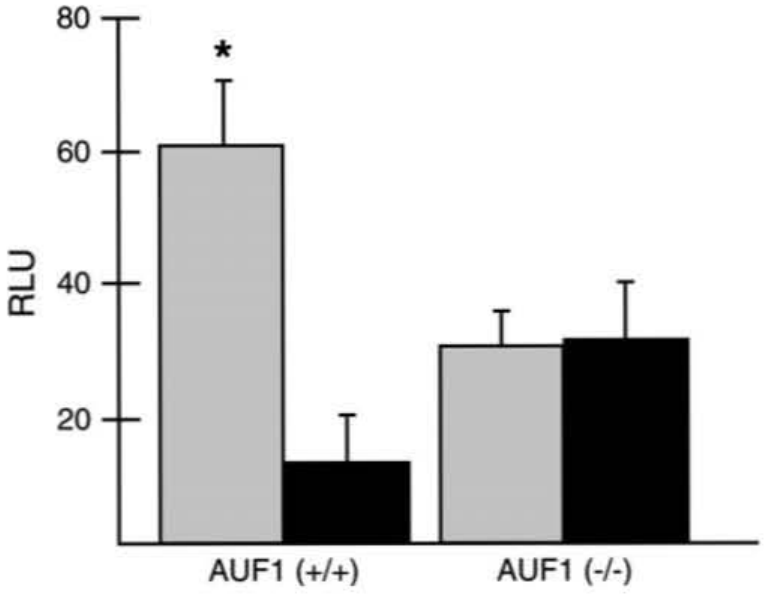


Figure II-6. The effect of TSA treatment on AUF1-dependent reporter gene activity. MEF-AUF1$^{+/+}$ and MEF-AUF1$^{-/-}$ cells were transfected with reporter plasmids pTK-rAT-Ren or pTK-rAT$_{MUT}$-Ren and treated with TSA (gray columns) or with vehicle (black columns). The results were normalized by subtracting values measured in cultures transfected with pTK-rAT$_{MUT}$-Ren from values obtained from cultures transfected with pTK-rAT$_{MUT}$-Ren and expressed as RLU. The lack of AUF1 in MEF-AUF1$^{-/-}$ cells resulted in a significant loss of the AT-rich DNA dependent repressor of AUF1 and a loss of the effect of TSA treatment. Star = $p \leq 0.05$; N = 4, ±SD.

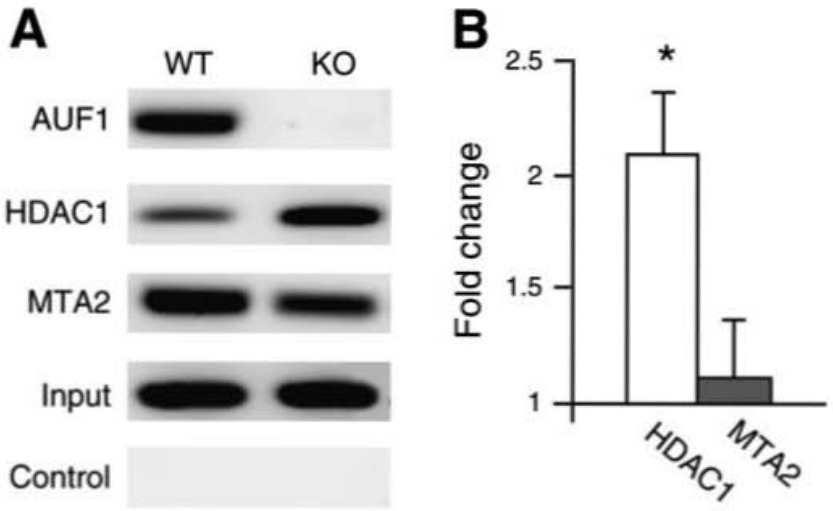
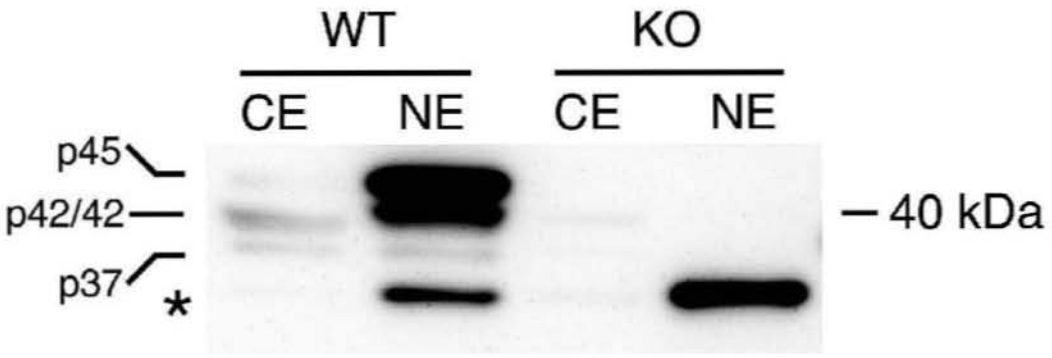
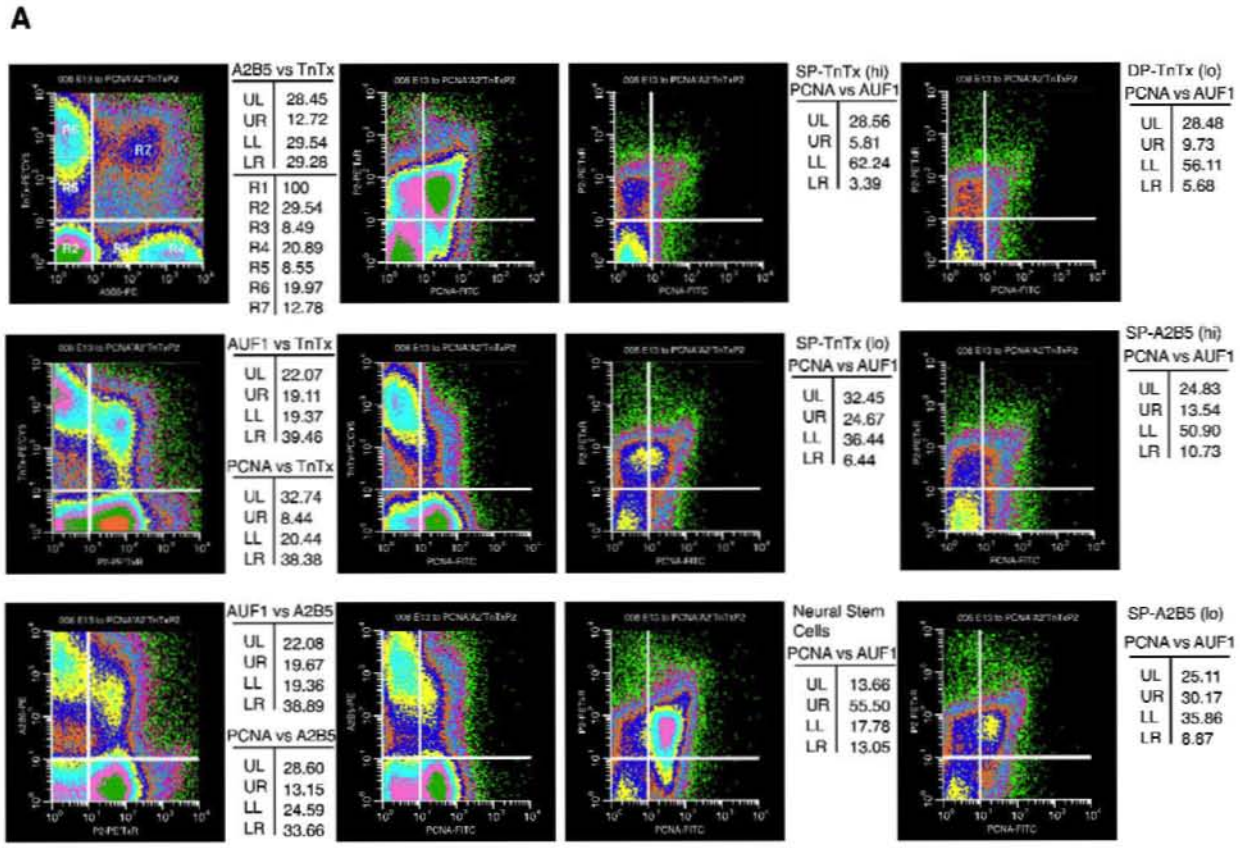


Figure II-7. AUF1 proteins interact with both the mAT^{ENK} region and the NuRD complex and control chromatin remodeling. Semiquantitative chromatin immunoprecipitation (ChIP) assay was performed using whole brain tissue from P2 wild type (WT) or AUF1 knockout (KO) mouse brains. A 274-bp DNA fragment containing the AT-rich regulatory region of the mouse ENK gene, or a negative control region was amplified from samples that were immunoprecipitated with anti-AUF1, anti-HDAC1, anti-SATB2 (Szemes et al. 2006) or anti-MTA2 antibodies, or normal rabbit serum as a negative control (not shown). As a negative control for AUF1-binding, we used a region in the GAPDH gene (control shown). After amplification by PCR, the product was subjected to agarose gel electrophoresis and DNA fragments were visualized by SYBR Safe staining (A). Images were collected using the Fuji 3000 LAS intelligent dark box equipped with a CCD camera, and band densities were quantified with the ImageGauge software. Raw values were normalized to the input and background, and then the fold change difference between KO to WT was calculated. Star = $p \leq 0.05$; N = 4. The average values from 3 independent experiments are shown with error bars indicating ± SD (B).

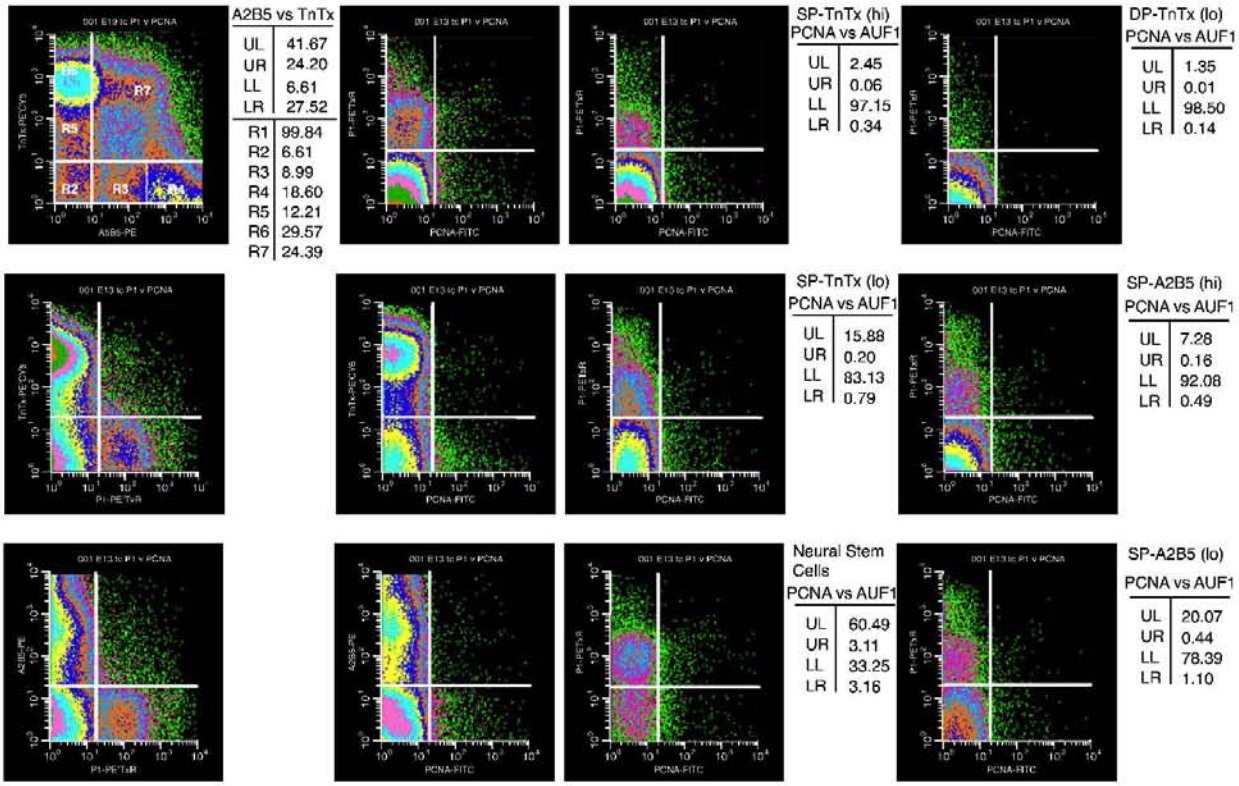


Supplementary Figure II-1. AUF1 isoforms as recognized by the commercial antibody. The commercial AUF1 antibody (Upstate, Waltham, MA) recognizes a non-specific ~30 kDa band (labeled with a star) in nuclear extracts prepared from P2 rodent cortices. CE: cytoplasmatic extract; NE: nuclear extract. WT: wild type; KO: AUF1 $^{-/-}$. Star indicates the recognized non-specific band.

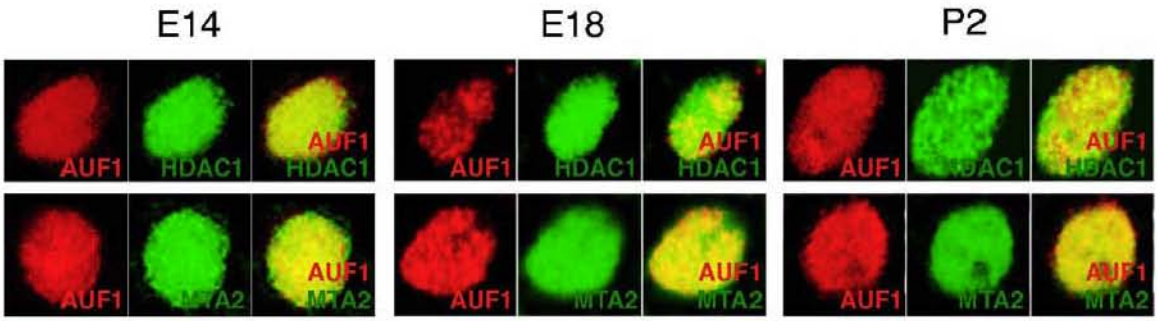


Supplementary Figure II-2. The phenotype of AUF1 expressing cells by FACS analysis. Bivariate dot density plots derived from FACS analysis showing that AUF1 is not expressed neither in tetanus toxin+ (Ttx+) mature neurons nor in developing (A2B5+) glia. Cells derived from E13 rat telencephalon (A) and from E19 rat cortex (B).

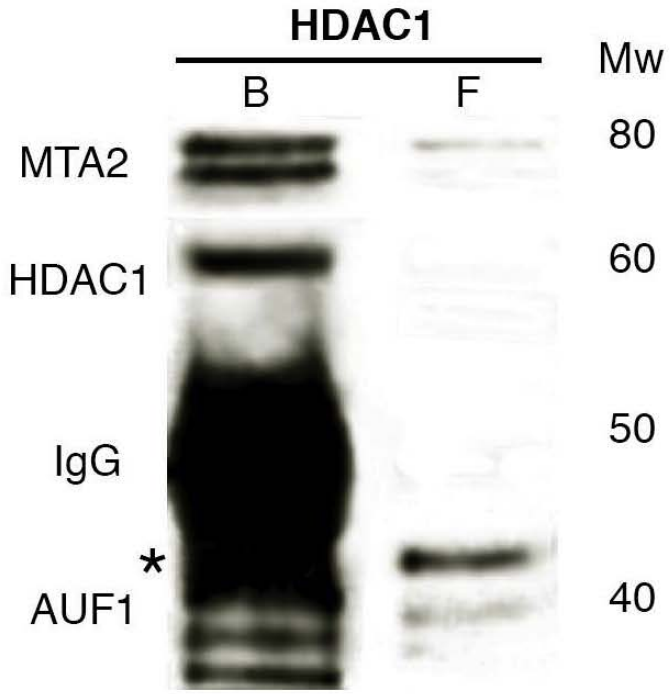
B



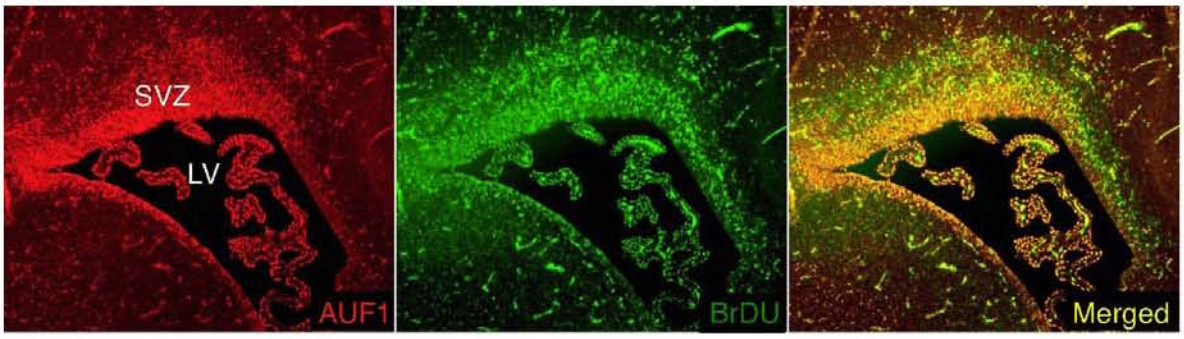
Supplementary Figure II-2. The phenotype of AUF1 expressing cells by FACS analysis Bivariate dot density plots derived from FACS analysis showing that AUF1 is not expressed neither in tetanus toxin+ (Ttx+) mature neurons nor in developing (A2B5+) glia. Cells derived from E13 rat telencephalon (A) and from E19 rat cortex (B).



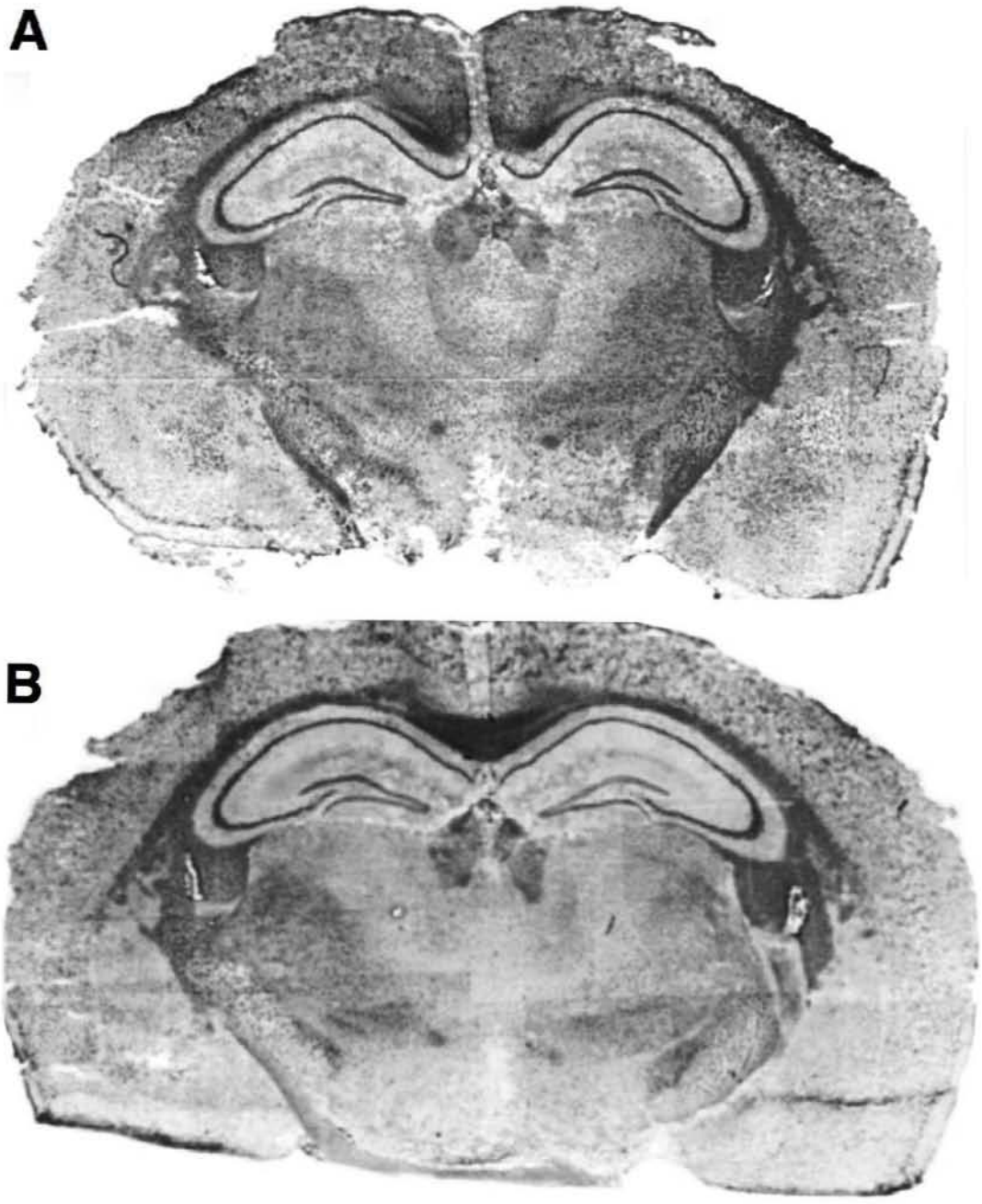
Supplementary Figure II-3. AUF1 (red) is co-expressed with both HDAC1 and MTA2 (green) at all ages tested. Double immunohistochemistry of coronal sections were derived from the E14, E18 and P2 cortex. Confocal images displaying the nuclear co-localization of AUF1 / HDAC1 and AUF1 / MTA2 in developing cortical neurons.



Supplementary Figure II-4. HDAC1 immunoprecipitation co-precipitates AUF1. B: bound fraction; F: supernatant; Mw: molecular weight in kDa. Star indicates AUF1, 45 kDa isoform.



Supplementary Figure II-5. AUF1 is expressed in BrdU+ proliferating cells. Double immunohistochemistry of coronal sections derived from E16 rat brain showing the partial co-localization of AUF1+ (red) and BrdU+ (green) cells. BrdU (50 mg/ kg) was injected 24 hrs prior to sacrificing animals. LV = lateral ventricle (with the developing choroid plexus).



Supplementary Figure II-6. AUF1 mutant animals have altered callosal development. A: coronal sections stained derived from adult AUF1 mutant (A) and wild type (B) animals with toluidine blue.

CHAPTER III

THE ROLE OF AUF1 IN THE ADULT HIPPOCAMPAL NEUROGENESIS

In the previous chapter we showed that AUF1 is involved in regulating cortical neurogenesis in the developing brain. Because some intrinsic factors have a conserved regulatory role on neurogenesis in the developing brain and in the adult brain [1], questions arise as to whether AUF1 is involved in regulating neurogenesis in the adult brain. Previous studies have shown that AUF1 is expressed in the nuclei of neuronal cells in the developing rat cortex and in the adult mouse hippocampus [2]. Rabbit polyclonal AUF1 antibody that used in the previous studies does not cross-react with other polypeptides and reacts specifically for all four AUF1 isoforms [3]. The same antibody was kindly provided by Dr. R. Schneider (New York University, New York, NY) and used for immunohistochemical analysis in this thesis work. To address the potential role of AUF1 in adult *de novo* neurogenesis, I have determined the expression pattern of AUF1 in the adult DG and characterized the phenotype of AUF1 positive cells by using double immunohistochemistry. AUF1 was expressed by most, if not all, granule cells in the adult DG (Figure III-1A, D, G). Within the DG, AUF1+ cells were present in both the SGL and also in the GCL. The observed AUF1 immunoreactivity by cells located in the SGL suggested that these cells may be neuroblasts / young neurons. In order to characterize AUF1 immunoreactive cells in the SGL, I performed double immunohistochemistry using a doublecortin (DCX) and a TuJ1 antibody in combination with the AUF1 antibody. As expected, DCX+ cells (Figure III-1B) and TuJ1+ cells (Figure III-1E) were found mainly in the SGL [4,5] and significant proportion of these cells was also AUF1 immunoreactive (Figure III-1C, F). In

addition to the SGL, AUF1 immunoreactivity was also detected in the GCL where most of the cells are postmitotic granule neurons. To analyze the expression of AUF1 by granule cells, I again performed double immunohistochemistry. Using NeuN as a marker for mature neurons in combination with AUF1, I found that a majority of NeuN immunoreactive cells expressed AUF1 (Figure III-1, G-I). All the detected AUF1 immunoreactivity in the GCL was associated with NeuN positive cells, indicating that AUF1 is expressed by granule cells and not by astrocytes or other cell types. These results showed that AUF1 expression spans from neuroblasts / young neurons to mature granule neurons in the DG of the adult hippocampus.

These results suggested that AUF1 may be expressed by *de novo* neurons in the DG, therefore I injected BrdU intraperitoneally into rats (50 mg/kg, twice in a 12-hour interval) to label mitotically active cells and to trace the fate of BrdU-labeled cells. The rats were sacrificed either at 3 days or at 20 days after BrdU injection (N=3 for each group) and I analyzed the brains by BrdU / AUF1 double immunohistochemistry. As expected, BrdU+ cells were observed in the SGL and GCL of the DG (Figure III-2). At 3 days after BrdU injection, most BrdU labeled cells were present in the SGL and only a few of them were BrdU+/AUF1+ (Figure III-2A, inset). A subset of BrdU+ cells appeared in the GCL, but only a few cells were double positive for BrdU and AUF1 (Figure III-2A, arrows). At 20 days after BrdU injection, however, a substantial number of BrdU+ cells were located in the GCL and approximately 50% of BrdU+ cells also expressed AUF1 (Figure III-2B, arrows). These experiments showed that AUF1 is expressed by *de novo* neurons in the DG of the hippocampus in the adult brain, suggesting that AUF1 may be involved in regulating *de novo* neurogenesis in the adult brain.

To understand the possible involvement of AUF1 in adult hippocampal neurogenesis, I have analyzed the hippocampus of adult AUF1 mutant animals. AUF1 was inactivated using

homologous recombination [6] and we received brains for histological analysis from Dr. Schneider's lab. I prepared cytoplasmic and nuclear extracts from the whole $AUF1^{+/+}$ and $AUF1^{-/-}$ brains by the NE-PER nuclear cytoplasmic extraction reagents (Pierce, Rockford, IL). Twenty μg of each brain tissue extract was loaded and blotted on PVDF membranes (Invitrogen, Carlsbad, CA). Polyclonal rabbit-anti AUF1 antibody (Upstate Biotechnology, Charlottesville, VA) was used to detect AUF1. I analyzed images with a Fuji LAS-1000 Luminescent Image analyzer and Fuji Image Gauge software. Western blot analysis showed that AUF1 immunoreactivity was almost exclusively nuclear. As expected, expression of all the AUF1 isoforms were completely missing in the $AUF1^{-/-}$ brain (Figure III-3). GAPDH was used as a loading control.

I also analyzed $AUF1^{+/+}$ and $AUF1^{-/-}$ brains by immunohistochemistry. There were no AUF1 immunoreactive cells in the brain of AUF1 mutant animals (Figure III-4A, B). I performed NeuN immunohistochemistry and Hoechst histology to see if the cytoarchitecture is altered in the mutant brain. At this level of analysis, I found no apparent differences in the cytoarchitecture between wild type and mutant brains except that the overall size of the hippocampus, particularly the size of the $AUF1^{-/-}$ DG appeared smaller in the AUF1 mutant brain (Figure III-4 C-F).

For a more detailed analysis, I performed additional immunohistochemical analysis using Prox1 antibody, a specific marker for dentate granule cells (Covance, Denver, PA). Immunohistochemical reactivity was visualized in an Olympus IX-71 microscope and images were acquired using a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). In addition to the reduced size of the DG, I found there was large number of Prox1 positive cells scattered seemingly outside of the boundaries of the DG in the AUF1 mutant brain (Figure III-5).

The coronal sectional area was measured by Fuji Image Gauge software and compared between wild type and mutant brains. Also I quantified the number of Prox1+ cells that were outside of the apparent boundary of the DG.

The area of AUF1 mutant DG was significantly reduced compared to that of the wild type DG (Student t-test, $N = 6$, $p < 0.001$) (Figure III-6A). In addition to the reduced size of the DG, the cytoarchitecture of the DG was altered in mutant animals. The number of Prox1+ cells scattered outside of the boundary of the DG increased by age and the difference between $AUF1^{+/+}$ vs. $AUF1^{-/-}$ animals after adjusting for age was statistically significant (ANOVA, $N = 4$, $p = 0.022$) (Figure III-6B).

Because the reduced size of the DG can be the result of decreased proliferation, I compared the number of proliferating cells in the SGL of AUF1 wild type and mutant animals by using Ki67 immunohistochemistry. Although I observed decreased proliferation in the mutant brain (Figure III-7), the effect of AUF1 mutation on proliferation of adult hippocampal stem / progenitor cells appears to be dependent on age and / or on generation of mutant animals. My observation in the brain is in agreement with the findings of our collaborators in the skin and the immune system where the effect of AUF1 mutation is also age- and generation-dependent. Whether AUF1 plays a regulatory role in the proliferation, maturation, and also in the migration of *de novo* hippocampal neurons will be addressed in future experiments by analyzing AUF1 mutant animals of different age and generation.

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Chapter III Figures

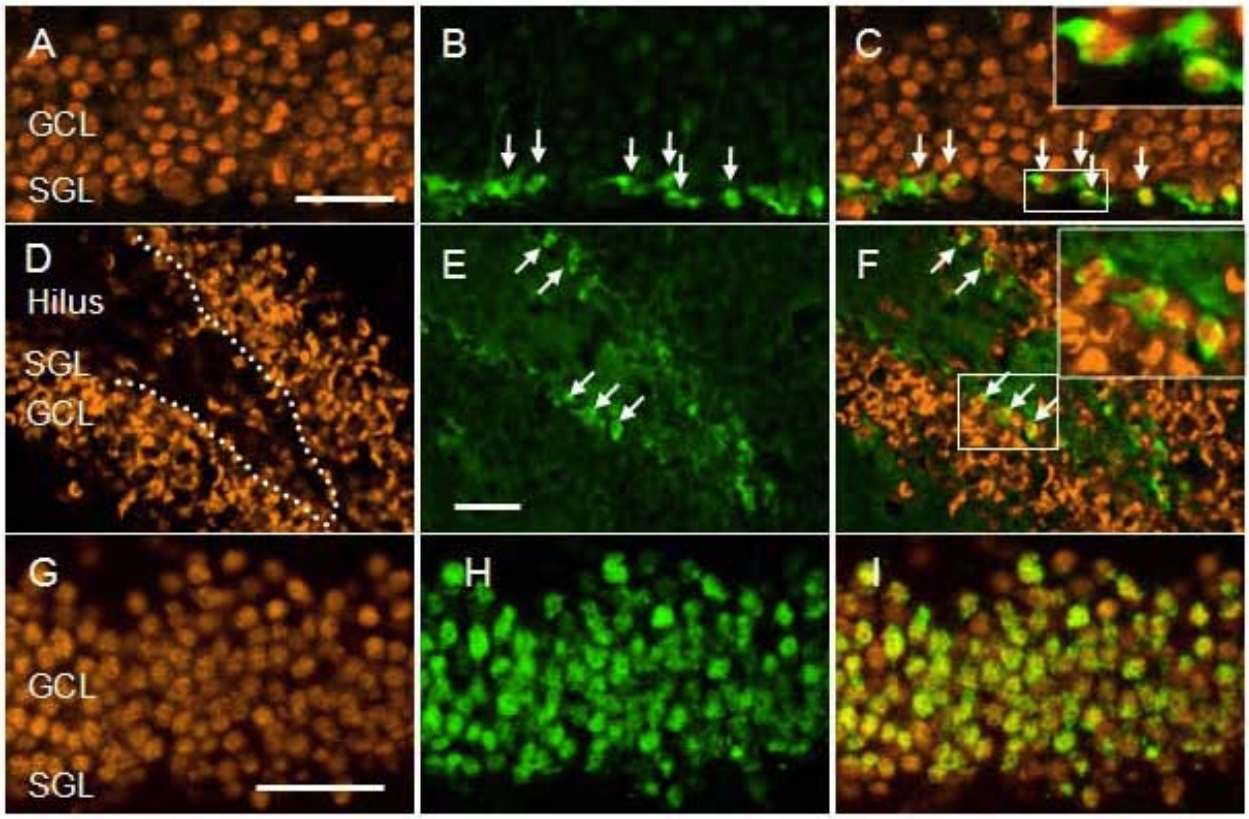

Figure III-1. The phenotype of AUF1 immunoreactive cells in the adult dentate gyrus (DG). AUF1 was expressed by most, if not all, granule cells in the adult DG. Within the DG, AUF1+ cells were present in both the subgranular layer (SGL) and also in the granule cell layer (GCL) (A, D and G). The phenotype of AUF1 expressing cells was determined by using double immunohistochemistry. A subset of AUF1+ cells in the SGL expressed DCX, a marker of neuroblasts / migrating neurons (A-C, arrows) and TuJ1, a marker of postmitotic differentiating neurons (D-F, arrows), suggesting that AUF1 is expressed in neuroblasts / differentiating young neurons. AUF1+ cells in the GCL coexpress NeuN, a marker of mature neurons (G-I). Scale bar = 50 μm

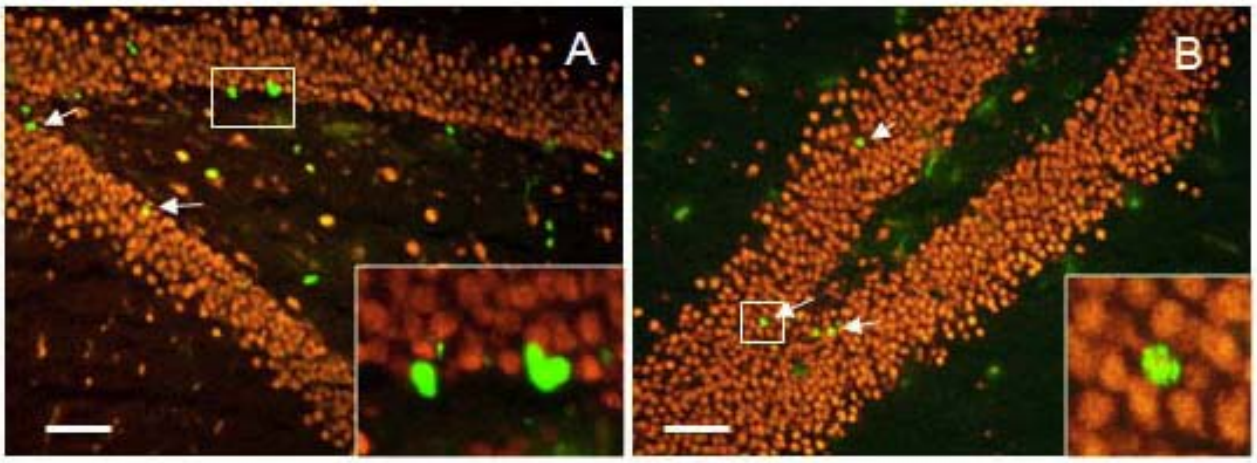

Figure III-2. AUF1 is expressed by *de novo* neurons in the adult dentate gyrus (DG). In order to determine whether AUF1 is expressed by *de novo* neurons in the DG, BrdU was injected intraperitoneally to rats to label mitotically active cells and trace the fate of BrdU labeled cells. Those rats were sacrificed either at 3 days or at 20 days after BrdU injection. BrdU / AUF1 double immunohistochemistry was subsequently performed. (A) At 3 days after BrdU injection, most of BrdU+ cells were localized in the subgranular layer (SGL). Only a few of them were BrdU+/AUF1+ (inset). Some of them appeared in the granule cell layer (GCL), but only a few cells were double positive for BrdU and AUF1. (B) At 20 days after BrdU injection, a substantial number of BrdU+ cells were located in the GCL and approximately 50% of BrdU+ cells expressed AUF1. Arrows indicate AUF1+/BrdU+ cells. Scale bar = 50 μm

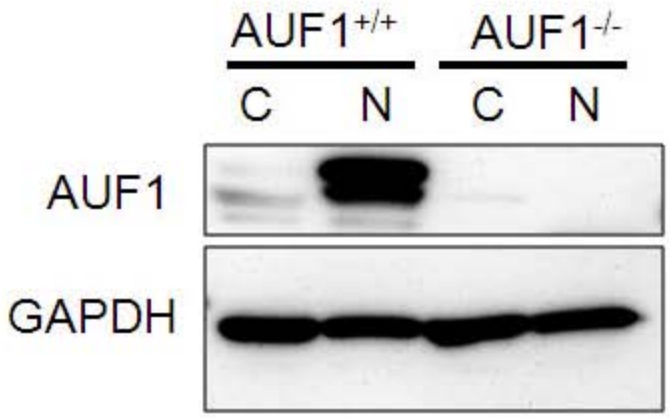

Figure III-3. AUF1 expression is missing in the AUF1$^{-/-}$ brain. Cytoplasmic (C) and nuclear extracts (N) were prepared from whole AUF1$^{+/+}$ and AUF1$^{-/-}$ brains. Twenty μg of extracts were loaded and blotted on PVDF membranes. Polyclonal rabbit-anti AUF1 antisera was used to detect AUF1. Western blot analysis showed that AUF1 immunoreactivity was almost exclusively nuclear. Expression of all the AUF1 isoforms were completely missing in the AUF1$^{-/-}$ brain. GAPDH was used as a loading control.

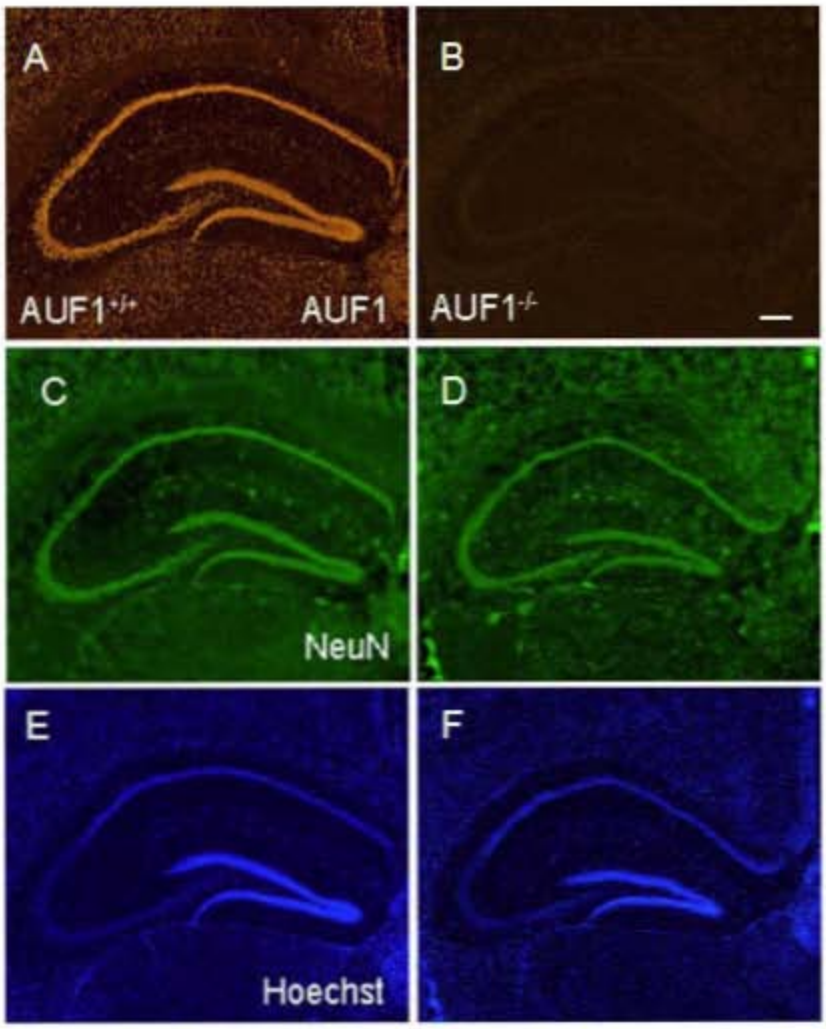

Figure III-4. Immunohistochemical analysis of the AUF1 mutant brain. Frozen adult $AUF1^{+/+}$ and $AUF1^{-/-}$ brains were cut and analyzed by immunohistochemistry. Polyclonal rabbit-anti AUF1 antisera (provided by Dr. Schneider) and monoclonal mouse-anti NeuN antisera were used. Sections were counterstained by Hoechst dye. There were no AUF1 immunoreactive cells in the brain of AUF1 mutant animals. NeuN immunohistochemistry and Hoechst histology showed no apparent differences in the cytoarchitecture between wild type and mutant brains. However, the overall size of the hippocampus, particularly the size of the $AUF1^{-/-}$ dentate gyrus (DG) appeared smaller than the $AUF1^{+/+}$ DG. Scale bar = 200 μm

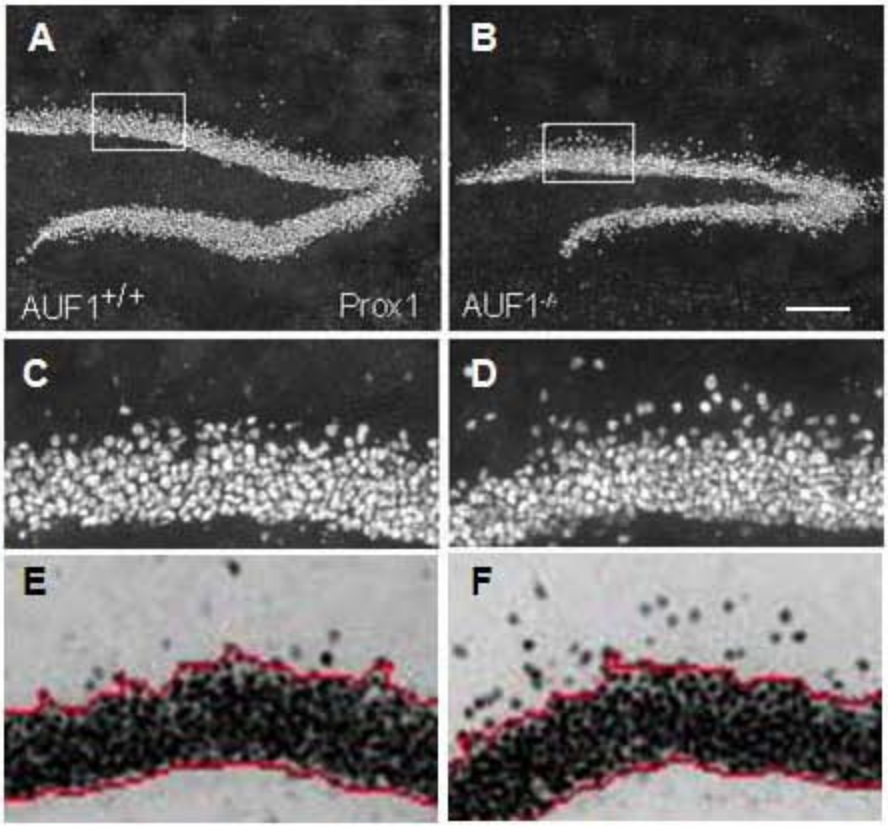

Figure III-5. Altered cytoarchitecture of the dentate gyrus (DG) of AUF1 mutant. For a more detailed analysis, dentate granule cells were immunostained using a specific marker, Prox1 antibody. Immunohistochemical reactivity was visualized and images were acquired. In addition to the reduced size of the DG, there was more number of Prox1 positive cells seemingly outside of the boundaries of the DG in the AUF1 mutant brain. The coronal sectional area was measured and compared between wild type and mutant brains. Also the numbers of Prox1+ cells present outside boundary (red lines in E, F) of the DG were analyzed. Scale bar = 200 μm

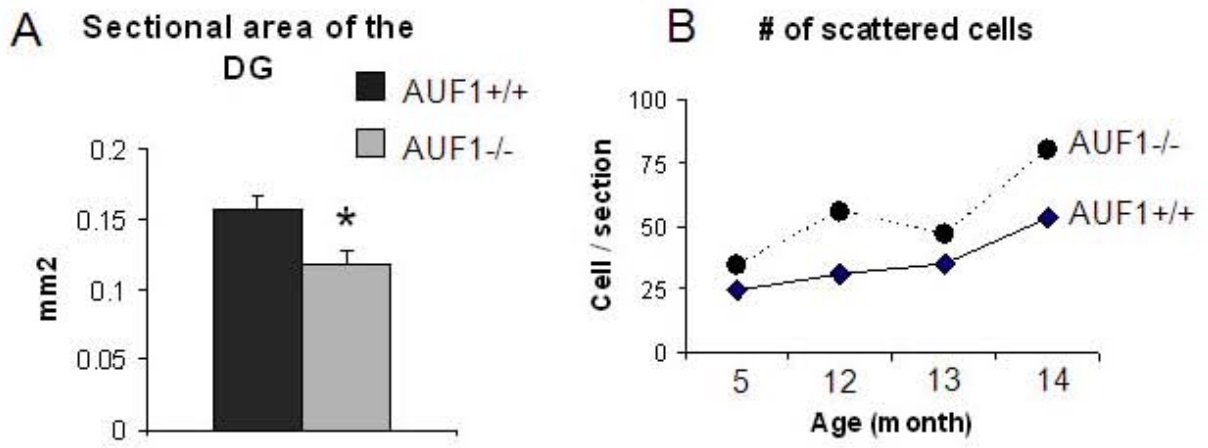

Figure III-6. Analysis of the altered cytoarchitecture of AUF1 mutant. (A) The area of AUF1 mutant DG was significantly reduced compared to that of the wild type DG (N = 6, $p < 0.001$). (B) The number of Prox1+ cells scattered outside of the boundary of the DG increased by age and the difference between $AUF1^{+/+}$ vs. $AUF1^{-/-}$ animals after adjusting for age was statistically significant (N = 4, $p = 0.022$).

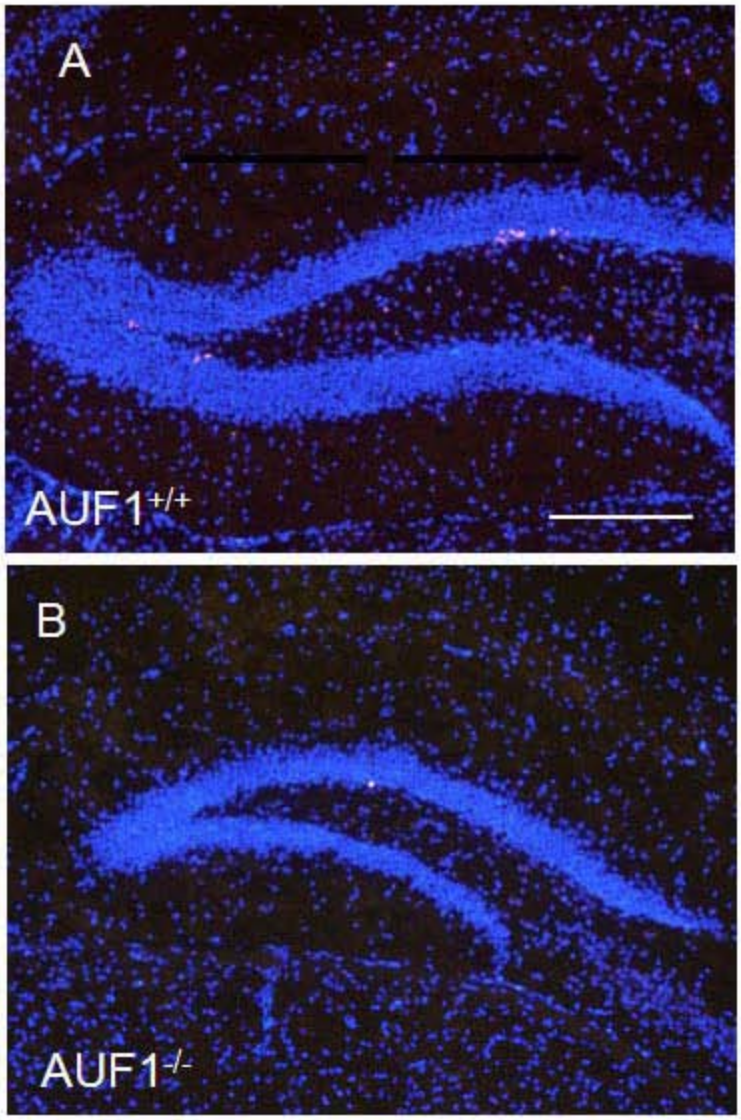

Figure III-7. Decreased number of proliferating cells in the dentate gyrus of AUF1 mutant. Ki67+ cells (orange) in the $AUF1^{+/+}$ brain (A) and $AUF1^{-/-}$ brain (B). Nuclei were stained with Hoechst dye (blue). Scale bar = 200 μm

CHAPTER IV

VEGF SIGNALING IS A MEDIATOR OF INCREASED *DE NOVO* HIPPOCAMPAL NEUROGENESIS IN THE INJURED RAT BRAIN

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Abstract

Increasing the rate of *de novo* hippocampal neurogenesis after traumatic brain injury (TBI) can be an important tool in neuroregenerative medicine. Because vascular endothelial growth factor (VEGF) is shown to regulate *de novo* hippocampal neurogenesis in various conditions, we tested whether VEGF signaling through its receptor Flk1 is involved in mediating neurogenesis after TBI. We found that Flk1 is expressed by both neuroblasts in the subgranular layer (SGL) and also by maturing granule neurons in the adult DG. Following TBI, the expression of VEGF, but not that of Flk1, was significantly and specifically upregulated in the ipsilateral DG in the injured rat brain. To directly test the role of VEGF and Flk1 in regulating TBI-induced neurogenesis, we delivered recombinant VEGF or SU5416, an inhibitor to Flk1, into the ipsilateral cerebral ventricle of animals that received fluid percussion TBI. We found that VEGF infusion significantly increased the number of *de novo* granule neurons in the DG compared to the number obtained from the control treated group. Following infusion with SU5416, the number of *de novo* granule neurons did not change significantly compared to the control treated group suggesting the involvement of additional VEGF receptor(s) and/or mechanism(s) in the process. Our results suggest that VEGF signaling is a part of the molecular signaling network that mediates *de novo* hippocampal neurogenesis after TBI and that VEGF predominantly mediates survival / differentiation of *de novo* granule neurons in the injured brain.

Keywords: Traumatic brain injury; Neurogenesis; Dentate gyrus; VEGF; Flk1; Osmotic pump

Introduction

It is now widely accepted that new neurons are generated in the adult mammalian brain throughout life. In normal conditions, this process occurs only in two regions of the brain. One is the subventricular zone of the lateral ventricle and the other is the subgranular layer (SGL) of the dentate gyrus (DG) of the hippocampus. Newborn cells from the SGL migrate to the granule cell layer (GCL) and many of the surviving neurons differentiate into granule cells (Altman and Das, 1965; Cameron et al., 1993). Some of the differentiated granule cells then integrate into the existing hippocampal circuitry and contribute to hippocampal functions in the normal adult brain (Saxe et al., 2006; Shors et al., 2001).

A large body of evidence shows that the basal rate of hippocampal *de novo* neurogenesis can be influenced both positively and negatively by various conditions and factors (Ming and Song, 2005). Various insults to the brain, such as ischemia and traumatic brain injury (TBI) can substantially increase the rate of *de novo* neurogenesis after a latent period (Chirumamilla et al., 2002; Dash et al., 2001). The initial cognitive dysfunction and memory impairment after TBI can be followed by significant functional recovery suggesting the existence of an endogenous repair process (Schmidt et al., 1999; Sun et al., 2007). An important component of this process may be the generation of new neurons in the SGL of the DG of the hippocampus (Dash et al., 2001; Sun et al., 2007).

Despite the importance of this potential repair process, the identity of molecules that mediate TBI-induced neurogenesis is currently not fully known. Several growth and neurotrophic factors, such as FGF-2, erythropoietin, and S100B, have been shown to contribute to an increase in the rate of neurogenesis after TBI (Kleindienst et al., 2005; Lu et al., 2005; Yoshimura et al., 2003). Recent experiments suggest that vascular endothelial growth factor (VEGF) is involved in mediating adult *de novo* hippocampal neurogenesis (Jin et al., 2002).

VEGF was first described as a vascular permeability factor, then as a major angiogenic factor, which promotes proliferation and survival of endothelial cells, and vascular formation (Dvorak et al., 1995). Increasing evidence, however, suggests that VEGF also has neurotrophic and neuroprotective effects in the CNS (Rosenstein et al., 2003; Svensson et al., 2002). VEGF exerts its function through several receptors, among them Flk1 (VEGF receptor 2 or KDR) is believed to mediate most neuron-specific effects of VEGF (Ogunshola et al., 2002; Sondell et al., 2000). Flk1 is a receptor tyrosine kinase that mediates the effects of its ligand through various downstream signaling molecules (Gerber et al., 1998). In the adult rodent brain, VEGF mediates the positive neurogenic effects of an enriched environment, physical activity, and antidepressants on the rate of adult *de novo* neurogenesis (Cao et al., 2004; Fabel et al., 2003; Warner-Schmidt and Duman, 2007).

In this study, we asked whether VEGF signaling through Flk1 is involved in mediating *de novo* hippocampal neurogenesis after TBI using the lateral fluid percussion model of TBI (LFP-TBI) in the rat. We characterized Flk1 expressing cells in the normal adult hippocampus by using double label immunohistochemistry for identifying the cell types that can be affected by VEGF. We then determined the effect of LFP-TBI on the expression of VEGF and Flk1 by Western blot analysis and ELISA. Finally, we altered VEGF signaling after LFP-TBI by delivering recombinant VEGF or SU5416, an inhibitor to Flk1, into the ipsilateral cerebral ventricle through chronically implanted cannula and analyzed the rate of proliferation and the number of *de novo* granule neurons.

Material and methods

Animals

Young Sprague-Dawley male rats (270-330 g) were used (Zivic Laboratories Inc., Pittsburgh, PA). Animals were handled according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at USUHS.

Lateral Fluid Percussion traumatic brain injury (LFP-TBI) model

Each rat was anesthetized with isoflurane, placed in a stereotactic apparatus and the dorsal scalp exposed through a midline cut under sterile conditions. A connector cannula hub was surgically placed through a 4.8 mm burr hole parasagittally over the right cerebral cortex at 2.5 mm lateral to the midline and 3.0 mm posterior to the bregma. A cannula was introduced through the hole until it abutted but did not violate the dural surface. This cannula was glued to the skull to keep it in place. A sterile saline filled catheter connected the cannula to the fluid percussion device. A fluid percussion pulse of 2.5 atm was administered by a pendulum-modulated fluid percussion (McIntosh et al., 1989). The cannula was then removed and the incision closed with surgical staples. Sham operated animals (control) were anesthetized and cannulated as LFP-TBI animals but the fluid percussion step was omitted. Rats were randomly assigned to two groups and rats in each group were sacrificed either 21 days post injury (dpi) or 28 dpi (Figure IV-1A).

Implantation of osmotic pump

One day after LFP-TBI, each rat was anesthetized and placed in a stereotaxic apparatus. Under sterile conditions, a short subcutaneous tunnel was created from the scalp incision to the midscapular area. Under sterile conditions, the dorsal scalp was re-exposed and a burr hole was created at 0.9 mm posterior to the bregma and 1.5 mm lateral to the midline. A 28 gauge intracerebroventricular (icv) cannula was implanted at a depth of 4.0 mm depth from the pial surface, targeting the right lateral ventricle. The position of the cannula was verified by histology after a tissue slide section was prepared (Supplementary Figure IV-1).

The osmotic pump (Alzet model 2002, Alzet, Cupertino, CA) was loaded with 10 μg/ml of recombinant rat $VEGF_{164}$ (Sigma, St. Louis, MO) or with 0.4 mM SU5416 (Calbiochem, San Diego, CA) in artificial cerebrospinal fluid (aCSF, consisting of 128 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 0.8 mM $MgCl_2$, 0.25 mM NaH_2PO_4, 21 mM $NaHCO_3$, 3.4 mM glucose, pH 7.4) containing 1% DMSO. The pump was then equilibrated with saline at 37 °C according to the manufacturer's instructions. The pump was placed in the subcutaneous pocket and connected to the implanted cannula. VEGF or SU5416 was delivered at the rate of 0.5 μl/hr for 13 days. The cell proliferation marker BrdU was added to VEGF or SU5416 to be delivered at a rate of 12 μg/day. As a control, vehicle (aCSF containing 1% DMSO) was infused along with BrdU by osmotic pump for the same amount of time periods (Figure IV-1B). All the concentrations were determined or modified based on the previous works (Pencea et al., 2001; Sun et al., 2003; Warner-Schmidt and Duman, 2007).

Tissue collection and processing

For the histological experiments, animals were placed under deep isoflurane anesthesia and transcardially perfused with cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde solution. Fixed brains were cryo-protected by immersing them in cold 15%

and 30% sucrose solution in PBS. Frozen brains were cut coronally in 20 μm sections with a cryostat (Cryocut 1800, Leica Microsystems, Bannockburn, IL). Tissue section slides were kept at -80 °C until use.

For Western blot analysis and ELISA, animals were decapitated under isoflurane anesthesia and brains were removed rapidly and kept in cold PBS. Under a stereomicroscope, the dentate gyrus, the remainder of the hippocampus, and the parietal cortex were microdissected from the ipsi- and the contralateral side to the injury, and the ipsilateral side of sham operated control. Tissue pieces were frozen by placing them on powdered dry ice and kept at -80 °C until use. Frozen tissue samples were quickly added to 10 volume of ice-cold lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Igepal, 0.5% deoxycholic acid, 1 mM AEBSF, 1 mM Na_3VO_4, 1 mM NaF) containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN). Cellular extracts were separated from tissue debris by centrifugation, and protein concentration was estimated by using the Protein Assay Kit (BioRad, Hercules, CA) according to the manufacturer's instructions. Cellular extracts were stored at -80 °C until use for Western blot analysis and ELISA.

Immunohistochemistry

Sections were hydrated with PBS and tissue antigens were retrieved by incubating them in 10 mM citrate buffer (pH 6.0) at 80 °C for 30 min followed by cooling down to room temperature (RT). The sections were rehydrated with PBS, permeabilized with 0.5% Triton X-100 in PBS and blocked in PBS containing 5% normal goat serum (NGS) and 5% bovine serum albumin at RT for 1hr each. Primary antibodies were diluted in 0.5% Triton X-100 in PBS containing 5% NGS and incubated on the slides for one or two nights in a humid chamber at 4 °C. Primary antibodies used in this study were mouse anti-Flk1 (1:200, Santa Cruz

Biotechnology, Santa Cruz, CA), rabbit anti-Prox1 (1:5000, Covance, Denver, PA), and rabbit anti-Doublecortin (1:200, Cell Signaling Technology, Beverly, MA). After being washed with PBS, secondary antibody (Alexa Fluor, 1:5000, Invitrogen, Carlsbad, CA) was applied for 1hr at RT, stained for 2 min with 1 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) for nuclear counterstaining, and mounted with anti-fading media (Vectashield, Vector Laboratories, Burlingame, CA).

BrdU immunohistochemistry

To label mitotically active cells and trace the fate of those cells after TBI, BrdU (Sigma, St. Louis, MO) was injected daily intraperitoneally (i.p., 50 mg/Kg, dissolved in sterile saline in volume of 0.2 – 0.5 ml) from 6 dpi to 10 dpi (Figure IV-1A). For immunofluorescent detection of BrdU, sections were treated with 2 N HCl at 37 °C for 30 min to denature DNA followed by the washing with 0.1 M borate buffer (pH 8.5). Mouse anti-BrdU (1:100, BD Biosciences, San Jose, CA) was used as a primary antibody. Further immunohistochemical procedures were same as above.

Histological data acquisition and analysis

Immunohistochemical reactivity was visualized in an Olympus IX-71 microscope and images acquired using a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI). Images were pseudocolored and superimposed using either Adobe Photoshop or the image analysis software in the Biomedical Instrumentation Center (BIC) at USUHS.

De novo neurons in the SGL and GCL were quantified by counting positive cells in all collected sections cut at the level of -2.5 mm to -3.5 mm of Bregma. A total of four to eight sections per brain were selected, processed, and analyzed.

Western Blot

Twenty μg of protein were mixed with sample buffer (NuPage LDS Sample Buffer, Invitrogen, Carlsbad, CA) and boiled for 5 min at 100 °C in a thermo-mixer. The samples were loaded on to a SDS-polyacrylamide gel and electrophoresis was performed at 100 V in a NuPAGE mini gel unit (Invitrogen, Carlsbad, CA) for 90 min. After electrophoresis, separated proteins were transferred to PVDF membrane using a Novex electroblotter with NuPAGE transfer buffer. After the transfer, PVDF membrane was blocked in blocking buffer consisting of 1% casein (BDH Laboratory, Poole, UK) in PBS which was followed by incubation in anti-VEGF antibody (1:1000, Lab Vision, Fremont, CA) or anti-Flk1 antibody (1:500, Lab Vision, Fremont, CA) in antibody solution (0.5% casein, 0.05% Tween-20 in PBS) overnight at 4 ^{o}C. The membranes were washed 3 times (15, 5, 5 min each) in PBST (0.05% Tween-20 in PBS). This was followed by incubation in horseradish peroxidase (HRP) conjugated secondary antibody diluted in antibody solution for 1hour at RT. After 4 wash cycles in PBST, immunoreactive proteins were visualized using SuperSignal ECL detection system (Pierce, Rockford, IL). Images were taken and band density was quantified using a Fuji digital camera (Image Reader LAS-3000 and ImageGauge V4.22, Fujifilm, Stamford, CT). Membranes were stripped (Re-Blot Plus, Chemicon, Temecular, CA) and reprobed with a GAPDH antibody (1:1000, Novus, Littleton, CO) as a loading control.

Enzyme linked immunosorbent assay (ELISA)

Total protein (125 μg) from each sample was diluted with PBS for a final volume of 100 μl and acidified to pH ~2.6 with 1 N HCl. After 15 min at RT, the acidified samples were neutralized with 1 N NaOH. Acidification and following neutralization increases the quantity of detectable neurotrophic factors in extracts of CNS tissues (Okragly and Haak-Frendscho, 1997; Shetty et al., 2005). The samples were assayed for VEGF using a rat VEGF ELISA kit according to the manufacturer's instructions (DuoSet, R & D systems, Minneapolis, MN). Using this kit, the concentration of VEGF in tissue extracts can be quantified within the range of 16 ~ 1000 pg/mL. Values were expressed as picogram of VEGF per microgram of total protein.

Statistical analysis

One-way ANOVA followed by a Tukey's post-hoc test was used for the intraventricular infusion experiment and a Student's t-test was used for the rest of the experiments. Data were expressed as the mean ± SD. p values of < 0.05 were considered significant.

Results

Flk1 is expressed in neuroblasts / young neurons and postmitotic granule neurons in the adult dentate gyrus

Previous studies have shown that VEGF regulates *de novo* neurogenesis in the adult brain (Jin et al., 2002). VEGF signaling in the CNS is also predominantly mediated by Flk1 (Ogunshola et al., 2002; Sondell et al., 2000). In order to determine whether VEGF induced signaling through the Flk1 receptor could be involved in mediating adult *de novo* hippocampal neurogenesis in the injured brain, we first analyzed the expression of Flk1 in the normal adult DG of the hippocampus and determined the differentiation stage of Flk1+ cells. Flk1 was expressed by cells in the subgranular layer (SGL), granule cell layer (GCL) and in the hilus of the DG (Figure IV-2). The observed Flk1 immunoreactivity by cells located in the SGL suggested that these cells may be neuroblasts / young neurons. In order to characterize Flk1 immunoreactive cells in the SGL, we performed double immunohistochemistry using a Doublecortin (DCX) antibody in combination with the Flk1 antibody. As expected (Nacher et al., 2001), DCX+ cells were found mainly in the SGL and significant proportion of these cells was also Flk1 immunoreactive (Figure IV-2). In addition to the SGL, Flk1 immunoreactivity was also detected in the GCL where most of the cells are postmitotic granule neurons. To analyze the expression of Flk1 by granule cells, we again performed double immunohistochemistry. Using Prox1 as a marker for postmitotic dentate granule neurons (Liu et al., 2000) in combination with Flk1, we found that a majority of Prox1 immunoreactive cells expressed Flk1. Importantly, all the detected Flk1 immunoreactivity in the GCL was associated with Prox1 positive cells, strongly suggesting that Flk1 is expressed by granule cells in the GCL and not by other cellular elements such as glia or endothelial cells. In order to determine whether Flk1 expression could be altered by LFP-TBI, we repeated the same immunohistochemical procedures on the LFP-TBI DG of the

hippocampus. We did not detect any observable change in Flk1 immunoreactivity after TBI (data not shown). These results showed that Flk1 is expressed by neuroblasts / young neurons and also by postmitotic granule neurons in the DG of the hippocampus, and therefore these cells could respond to VEGF signaling.

The expression of VEGF but not that of Flk1 is upregulated after TBI

VEGF mediates *de novo* hippocampal neurogenesis induced by physical activity and enriched environment (Cao et al., 2004; Fabel et al., 2003). To test our hypothesis that VEGF signaling contributes to the observed increase in *de novo* hippocampal neurogenesis in the TBI brain (Chirumamilla et al., 2002; Dash et al., 2001), we first tested whether either VEGF or Flk1 protein levels, or both would change in the DG in response to TBI. For Western blot and ELISA analyses, we microdissected the DG, the hippocampi (minus DG), and the parietal cortices from the ipsi- and contralateral sides of injured, and the ipsilateral side of sham operated animals. The microdissected brain regions were individually processed for Western blot analysis using a VEGF specific antibody. The precision of microdissection was tested by analyzing the individual brain regions for Prox1 immunoreactivity, a specific marker of granule cells in the DG (Liu et al., 2000). The abundance of Prox1 immunoreactivity in the dissected DGs, the low levels or absence in the hippocampal and cortical samples validated the microdissection (Figure IV-3A). Western blot analysis showed that VEGF expression increased significantly in the injured (ipsilateral) DG compared to the sham or contralateral DG, but not in the hippocampus or cortex at 5 dpi. We found that VEGF expression further increased 7 dpi in the ipsilateral DG but in no other brain regions (Figure IV-3B). We verified the changes in VEGF levels measured by Western blot analysis by using ELISA (Supplementary Figure IV-2). We also measured Flk1 protein levels by Western blot analysis and found no significant differences among the various

experimental groups (Figure IV-3C). These results showed that there is significant increase in VEGF protein levels at 5 dpi and 7 dpi.

De novo *hippocampal neurogenesis increases after LFP-TBI.*

The number of proliferating cells and *de novo* neurons in the DG increases significantly after various insults to the brain (Gould and Tanapat, 1997; Liu et al., 1998; Parent et al., 1997). To verify that our LFP-TBI model produces the same effect, we analyzed the number of *de novo* neurons in the ipsilateral and contralateral DG of injured, and control (sham) brains. To label newborn cells we daily injected BrdU intraperitoneally from 6 dpi to 10 dpi. At 21 or 28 dpi, animals were sacrificed and processed for BrdU and Prox1 immunohistochemistry. As expected, BrdU+ cells were observed in the SGL and GCL of the DG (Figure IV-4A, B). Many of these cells were also positive for Prox1, a marker of granule neurons located in the SGL and GCL (Figure IV-4A, B). At 21 dpi, the number of Brdu+/Prox1+ *de novo* granule neurons in the ipsilateral DG increased approximately two-fold compared to that of sham operated animals (N = 5, p=0.018). When we compared the number of *de novo* neurons between the ipsi- and contralateral sides, we found that approximately 40% more new neurons were generated in the ipsilateral DG compared to the contralateral DG (N = 5, p=0.038) (Figure IV-4C). At 28 dpi, the trend and extent of differences, regarding the number of *de novo* neurons, among three groups (ipsi vs. contra vs. sham) were similar to those of 21 dpi, however due to large variations in cell numbers in the ipsilateral DG, the differences were not statistically significant (data not shown). These results demonstrate that there was a significant increase in the number of *de novo* neurons in the ipsilateral DG following TBI. This increase in the number of *de novo* neurons in the ipsilateral DG corresponds to the increase in VEGF expression in the injured ipsilateral DG. These results suggested that VEGF may be involved in mediating the TBI induced increase in the rate of *de novo* neurogenesis.

Altered VEGF signaling affects the rate of TBI induced neurogenesis

To directly test whether the rate of TBI induced neurogenesis is mediated by VEGF/Flk1 signaling, we infused recombinant rat $VEGF_{164}$ or the Flk1 inhibitor SU5416 along with BrdU intracerebroventricularly (icv) using an osmotic pump after LFP-TBI. The animals in the control group were infused with vehicle containing BrdU after LFP-TBI. Following the surgical procedures (Figure IV-1B), we quantified the number of *de novo* granule neurons (Prox1+/BrdU+) and the number of proliferating cells (BrdU+) in the ipsilateral DG (Figure IV-5 A-I). Following VEGF infusion, the number of *de novo* granule neurons increased significantly compared to the number obtained from the control group (N = 5, p=0.016). Following infusion with SU5416, the number of Prox1+/BrdU+ cells did not change significantly compared to the control group but it was significantly lower than the VEGF treated group (N = 5, p=0.004) (Figure IV-5J). When we plotted only the number of proliferating (BrdU+) cells and compared them, we found no statistically significant difference between the control and VEGF treated or control and SU5416 treated groups (Figure IV-5K). As expected, the only statistically significant difference in the number of BrdU+ cells we found was between the VEGF and SU5416 treated groups (N = 5, p = 0.012).

Discussion

De novo hippocampal neurogenesis can be a substantial component of the regenerative process after TBI (Dash et al., 2001; Sun et al., 2007). Here we demonstrate that VEGF signaling is a part of the molecular signaling network that links TBI to the regenerative attempt characterized by the increased number of *de novo* granule cells in the hippocampus.

Our finding that VEGF expression is only elevated in the ipsilateral DG but not in other brain regions of the TBI brain, including the ipsilateral hippocampus (minus DG), suggests that there is a selective regulatory mechanism of VEGF expression in the DG in response to brain insults. This may be a part of the molecular cascade that mediates injury induced *de novo* neurogenesis in the DG. Transiently elevated expression of VEGF restricted to the DG has been reported after systemic hypoxia and electroconvulsive seizures (Marti and Risau, 1998; Warner-Schmidt and Duman, 2007). The mechanism underlying how insults to the entire brain results in a very localized signaling event is currently not understood. VEGF expression itself is regulated by various factors in multiple steps. These include numerous transcription factors, cytokines and soluble factors, and different hormones that regulate the expression of VEGF at the transcriptional level (Pages and Pouyssegur, 2005). Some of these factors regulate VEGF levels by altering the translation initiation or the stability of VEGF mRNA (Levy et al., 1996).

The initial pathological changes after LFP-TBI are hemorrhage and acute cell death in the focal point of the injury (McIntosh et al., 1989). These acute symptoms are followed by progressive secondary damages observed in selectively vulnerable brain regions such as the hippocampus (Thompson et al., 2005). The secondary damages result from various pathophysiological conditions such as altered cerebral blood flow, release of excitatory neurotransmitters, recruiting immune cells, inflammation, astrogliosis, and upregulation of

various kinds of cytokines and growth factors (Thompson et al., 2005). Some of these conditions can contribute to the induction of VEGF gene expression. For example, cerebral hemorrhage and hypoperfusion as well as alterations in vascular integrity may change local blood flow resulting in local hypoxia or ischemia (McIntosh et al., 1989). Hypoxia is a well known inducer of VEGF expression by activation of VEGF gene transcription through HIF-1 (Forsythe et al., 1996). In addition, altered levels of cytokines and growth factors including IL-1β, IL-6, and TNF-α following TBI may affect the expression of VEGF (Ahn et al., 2004; Pages and Pouyssegur, 2005).

VEGF exerts its function through VEGF receptors, among them Flk1 is believed to be responsible for most neuron-specific effects of VEGF (Ogunshola et al., 2002; Rosenstein et al., 2003; Sondell et al., 2000). Flk1 is a membrane bound tyrosine kinase whose activation stimulates various intracellular signal transduction pathways such as the PI3K/Akt and MEK/ERK pathways (Gerber et al., 1998; Wu et al., 2000). The PI3K/Akt pathway mediates the neuroprotective function of VEGF (Kilic et al., 2006), while the MEK/ERK pathway influences proliferation of retinal progenitors (Hashimoto et al., 2006). For the neurogenic effect of VEGF, VEGF can act directly on Flk1-expressing neural progenitor cells and/or differentiating young neurons. For example, VEGF expression increased after transient forebrain ischemia followed by enhanced cell proliferation in the DG but no changes in sectional areas of cerebral vessels or vascular densities in the DG (Kawai et al., 2006). Furthermore, in the avascular chicken retina, VEGF acts through the Flk1 present on progenitor cells to influence cell proliferation and commitment (Hashimoto et al., 2006). Alternatively, VEGF can exert its effects on neurogenesis via other non-neuronal cell types in the brain. It should be noted that studies indicate an intimate and symbiotic but not fully understood relationship between endothelial cells and neural stem cells in the “neurogenic niche” of the hippocampus (Palmer et al., 2000). Endothelial cells provide various signals that can regulate the rate of proliferation of neural stem cells and the

survival of *de novo* neurons in the DG (Louissaint et al., 2002; Shen, et al., 2004). Accordingly, VEGF can activate vascular endothelial cells and/or induce *de novo* angiogenesis, which in turns supports *de novo* neurogenesis. In addition, delivery of exogenous VEGF or VEGF gene transfer into the brain can stimulate angiogenesis as well as neurogenesis (Cao et al., 2004; Sun et al., 2003). Also, upregulated VEGF following injury to the CNS can activate astrocytes that in turn play critical roles on neurogenesis (Krum and Khaibullina, 2003; Song et al., 2002)

Our immunohistochemical results demonstrated that Flk1 is expressed in neuroblasts and postmitotic granule cells, suggesting that VEGF can act on those cells. Based on the proposed sequence of cell types in adult hippocampal neurogenesis, Flk1 expressing cells correspond to the stage 3 to stage 6 (Kempermann et al., 2004). The cell types in these stages are lineage determined, have limited self-renewal, are postmitotic, and are undergoing differentiation. This suggests that in adult hippocampal neurogenesis, VEGF is involved in mediating the differentiation / maturing process of young neurons rather than proliferation of neural stem / progenitor cells. Our experimental results derived from the chronic icv infusion of VEGF are in agreement with this idea. We observed significantly higher number of *de novo* neurons (Prox1+/BrdU+) after VEGF infusion than in the control treated group, whereas the number of proliferating (BrdU+) cells was not significantly different. The ratio of the number of *de novo* neurons to the number of total newborn cells (the number of BrdU/Prox1 double positive cells divided by the total number of BrdU positive cells) in the VEGF infused group was significantly higher than that in the control group. (77.9 ± 4.8% and 59.1 ± 17.1% respectively, N = 5, $p = 0.046$). This is important because it indicates that there is a “dose-dependent” effect of VEGF and that additional exogenous VEGF treatment can be beneficial for *de novo* neurogenesis in the injured brain.

The observed net increase in the numbers of *de novo* neurons after exogenous VEGF treatment in the injured brain can be the result of increased survival and/or a decrease in the number of apoptotic cells. Neurogenesis is defined as the series of developmental steps that

include the proliferation of neural stem/progenitor cells, neuronal fate determination, differentiation of immature neurons, and the functional integration of neuronal progeny into neuronal circuits (Ming and Song, 2005). During this processes, a significant proportion of newborn cells die within a few days after final division (Biebl et al., 2000; Kempermann et al., 1997). Most of the surviving newborn cells, however, differentiate into neurons and remain stable up to 11 months after birth (Kempermann et al., 2003). TBI can influence the survival or apoptotic death of newborn cells. It has been shown that fewer newborn cells survived in the injured DG than the sham operated DG (Sun et al., 2007). If the decreased survival rate of newborn cells after TBI stems from a limited amount of available neurotrophic support, providing neurotrophic factor could help newborn cells to survive and progress further in neuronal differentiation. Our results support this notion that VEGF can contribute to the TBI-induced neurogenesis by enhancing cell survival / neuronal differentiation through providing neurotrophic support rather than through increasing cell proliferation.

We found that chronic infusion with Flk1 inhibitor SU5416 failed to reduce the number of *de novo* neurons below control values in the injured adult brain. When we plotted only the number of BrdU+ cells, we observed that same SU5416 treatment decreased the number of proliferating cells in the DG as compared to the numbers obtained from control (injured but untreated animals). However, the difference was not significant ($p < 0.1$). This finding suggests that the concentration of SU5416 was insufficient to block the effect of endogenous VEGF (which was significantly increased in the injured DG, see Figure IV-3A, B). However, this is unlikely because we observed a statistically significant difference between numbers of BrdU+ cells in VEGF and SU5416 infused animals. More likely, there may be another receptor and/or Flk1-independent mechanism(s) involved in mediating the de novo neurogenesis and proliferation in the injured brain.

In the present study we focused on VEGF signaling as an underlying mechanism of TBI induced neurogenesis. One may raise a question whether the TBI-induced neurogenesis is

functional and linked to cognitive recovery. Although we have not assessed the behavioral effect of VEGF treatment in this series of experiments, there have been studies showing that the increased neurogenesis after TBI is closely associated with functional recovery. New neurons generated in the DG of adult rat brain following TBI are capable of receiving synaptic input and projecting axons along the mossy fiber pathway to the CA3 region of the hippocampus (Emery et al., 2005). Importantly, this anatomical integration is temporally associated with innate cognitive recovery (Emery et al., 2005; Sun et al., 2007). It is well known that humans and animals at younger ages show better cognitive recovery than older animals after TBI (Hoane et al., 2004; Marquez de la Plata et al., 2008). Recently it has been demonstrated that juvenile rats produce more neurons in the DG than adult rats after TBI (Sun et al., 2005). These results suggest that higher neurogenic response to injury may underlie the better cognitive recovery in young animals compared to the aged counterparts following TBI. Moreover, elimination of cell proliferation in the DG by irradiation reduces cognitive recovery in adult rats after TBI (Richardson et al., 2007). These results suggest that TBI-induced neurogenesis is crucial for functional recovery from the initial cognitive impairment and memory deficit after TBI. However, it should be noted here that increased *de novo* neurogenesis is not always beneficial. For example, in the pilocarpine-induced seizure model, the increased number of new granule cells in the DG migrates to aberrant locations and thus may worsen the outcome (Parent, 2003; Scharfman et al., 2000). Further animal behavioral experiments, evaluating cognitive recovery after TBI using our current paradigm, will provide better understanding for the role of VEGF signaling on TBI-induced neurogenesis as a part of endogenous repair mechanism following neurotrauma. It will be very interesting to determine that how TBI-induced apoptotic pathway (Liou et al., 2003) is affected by neurotrophic effect of VEGF.

It has been shown that VEGF signaling has neurogenic and neuroprotective functions in other brain injury animal models such as ischemia (Jin et al., 2000; Kawai et al., 2006). In addition, VEGF has a more extended role for adult neurogenesis. VEGF plays a role as a

molecular mediator of various external stimuli (the effect of the environment) on the rate of neurogenesis and behaviors. The neurogenic effect of enriched environment, physical activity, and antidepressants is mediated by VEGF, which is associated with behavioral outcome (Cao et al., 2004; Fabel et al., 2003; Warner-Schmidt and Duman, 2007). Our data extend these findings by showing that VEGF can act as an underlying molecule of TBI-induced neurogenesis. Together, those previous studies and ours raise possibility of VEGF signaling as a common downstream mediator of various external stimuli and hippocampal neurogenesis.

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CHAPTER IV Figures

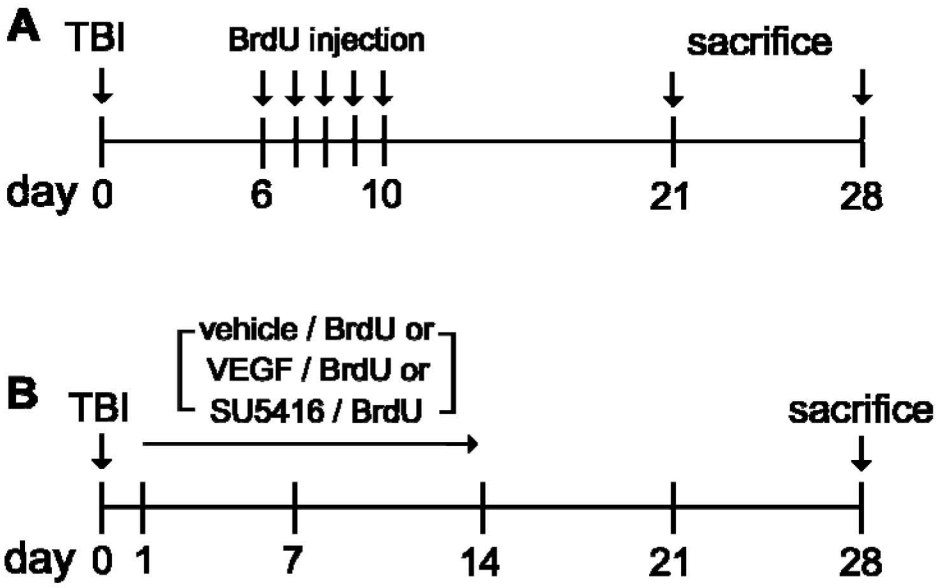


Figure IV-1. Experimental design and timetable of the surgical procedures. (A) To determine the effect of TBI on neurogenesis, BrdU was daily injected intraperitoneally from 6 days post injury (dpi) to 10 dpi. Rats were randomly assigned to two groups and rats in each group were sacrificed either 21 dpi or 28 dpi. (B) For the intracerebroventricular (icv) infusion experiment, one day after TBI, rats were randomly assigned to three groups. Rats in each group were subjected to a continuous icv infusion of either vehicle / Brdu (control) or VEGF / BrdU or SU5416 / BrdU using an osmotic pump for 13 days. Infusion was discontinued and rats were allowed to live for 14 days until they were sacrificed.

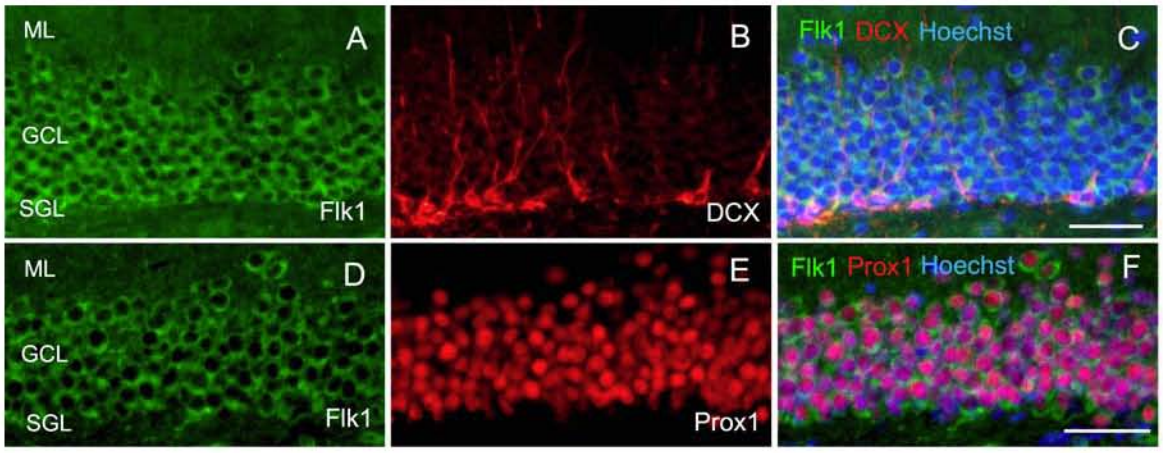


Figure IV-2. Flk1-expressing cells are present in the dentate gyrus (DG) of the adult rat. Flk1 immunoreactive cells are localized in the subgranular layer (SGL) and the granule cell layer (GCL) of the DG. Neuroblasts / young neurons (DCX+) mainly populate in the SGL. Double immunofluorescence shows that a subset of DCX+ cells in the SGL express Flk1. Majority of postmitotic granule neurons (Prox1+) in the GCL express Flk1. Sections were counter stained with Hoechst dye (blue). ML = molecular layer, Scale bar = 50 μm.

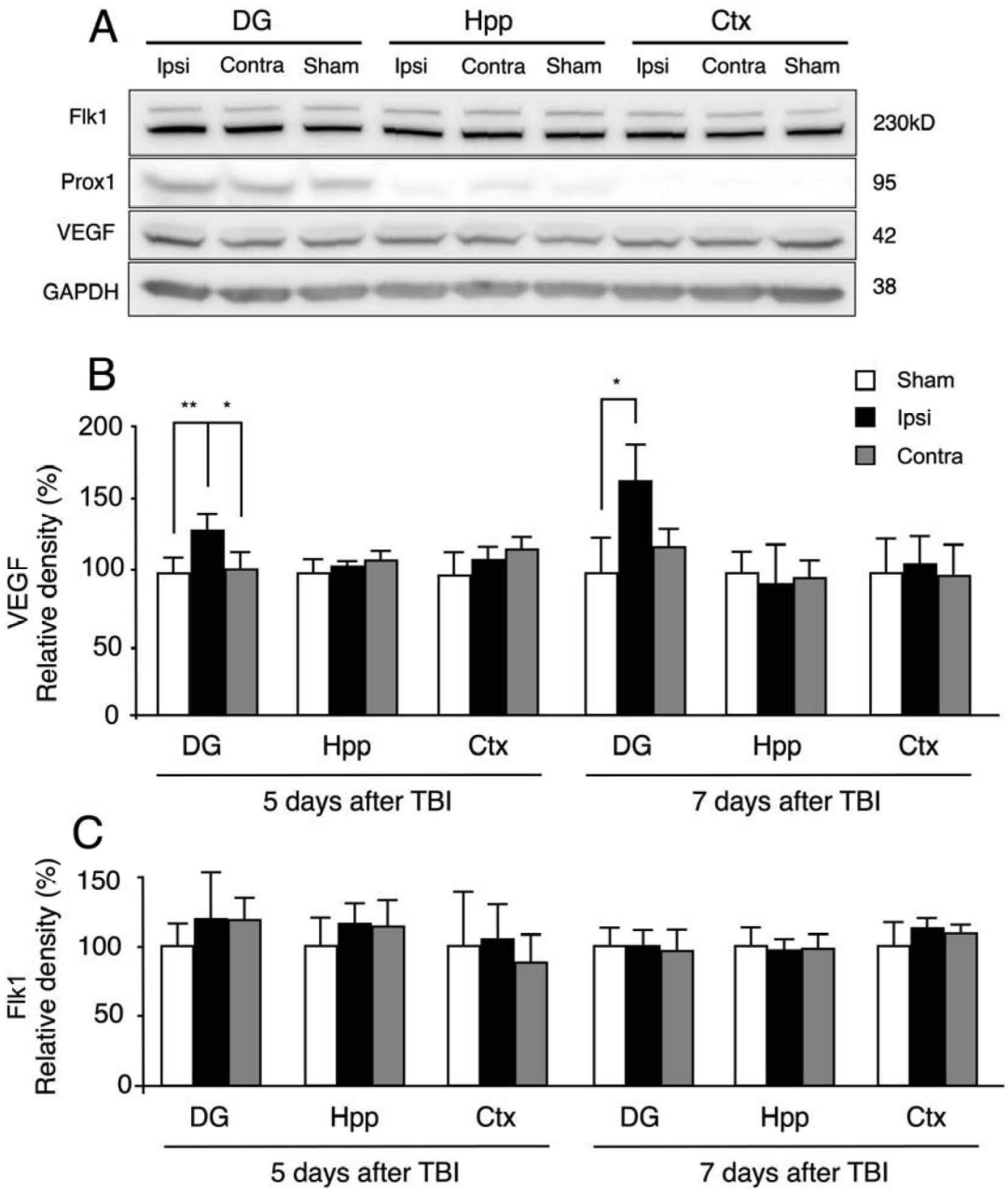


Figure IV-3. Traumatic brain injury alters the expression of VEGF. (A) A representative Western blot analysis showing the abundance of Flk1, Prox1, and VEGF. GAPDH was used as a loading control. Molecular weights are indicated on the right side. The presence of Prox1 immunoreactivity in the dentate gyrus (DG) and the low levels or absence in the hippocampus (Hpp) and cortex (Ctx) validate the microdissection of the DG. (B) Quantitative analyses of VEGF expression as measured by Western blot analysis. Relative band densities from each group were averaged and plotted. VEGF expression in the ipsilateral (Ipsi) DG of TBI brain increased significantly 5 and 7 days post injury (dpi). (C) Quantitative analyses of Flk1 expression as measured by Western blot analysis. There were no significant differences among the various experimental groups in the level of Flk1 expression. Data represent averages of duplicated experiments. Error bars indicate SD. N = 5 for 5 dpi group and N = 4 for 7 dpi group. *$p < 0.05$, **$p < 0.01$, Contra = contralateral

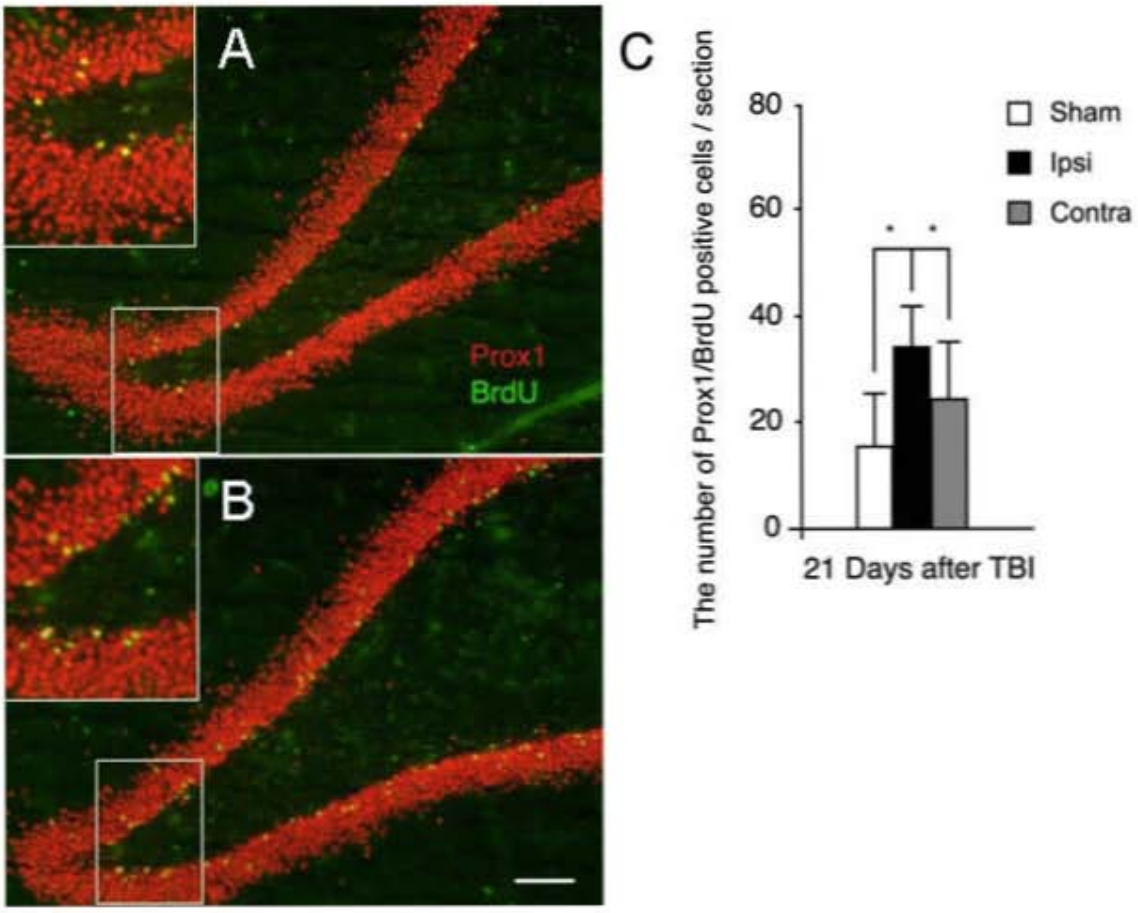


Figure IV-4. Traumatic brain injury increases the number of *de novo* granule neurons in the dentate gyrus (DG). Representative double immunofluorescent staining for Prox1 and BrdU in the DG of sham operated (A) and injured brain (B) at 21 days post injury (dpi). (C) Quantitative analysis of *de novo* granule neurons (Prox1+/BrdU+) in the DG among ipsilateral sham operated brain (sham), ipsilateral injured brain (ipsi), and contralateral injured brain (contra) at 21 dpi. Data represents the average number of double (Prox1+/BrdU+) immunoreactive cells per section. 4 ~ 8 sections from each animal were used. Error bars indicate SD. Scale bar = 100 μm. N = 5, *p < 0.05

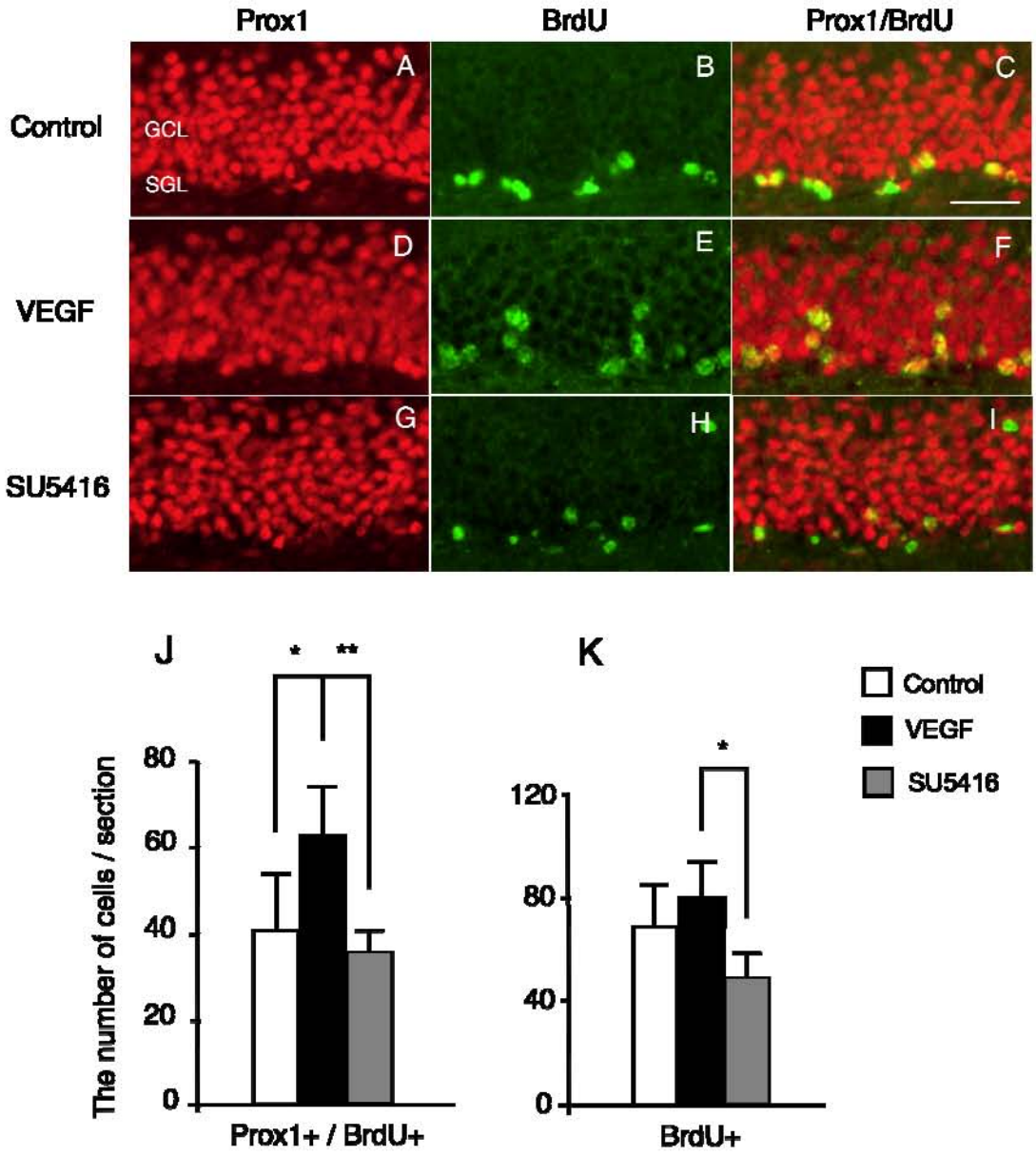
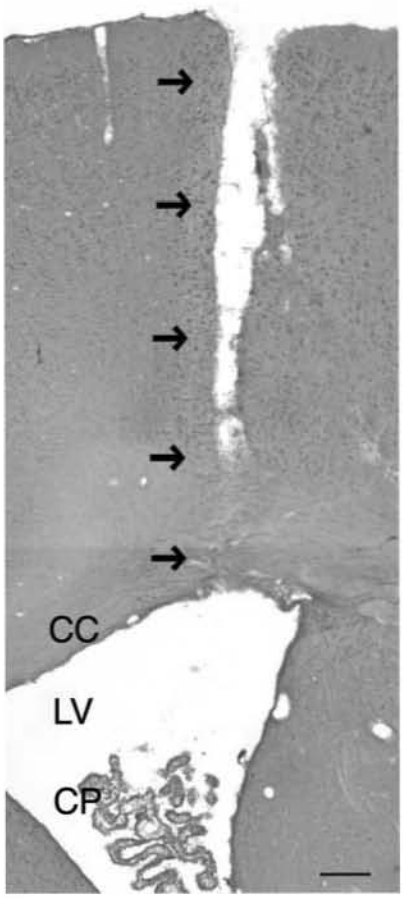
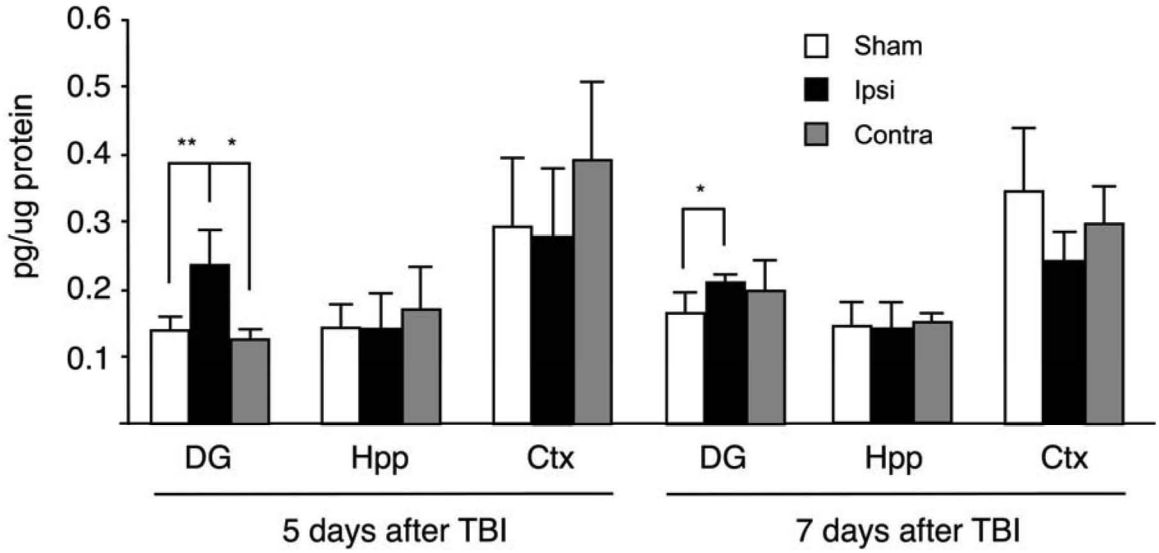


Figure IV-5. Administration of recombinant VEGF affects the rate of *de novo* neurogenesis in the injured brain. Representative double immunofluorescent staining for Prox1 (A, D, G) and BrdU (B, E, H) in the dentate gyrus (DG) of animals that were infused either vehicle (control) (A-C) or VEGF (D-F) or SU5416 (G-I) after TBI. Quantitative analysis of *de novo* granule neurons (Prox1+/BrdU+) or newborn cells (BrdU+) in the DG of three animal groups (J, K). Data represent the average number of double (Prox1+/ BrdU+) or total BrdU+ cells per section. 6 sections from each animal were used. Error bars indicate SD. GCL = granule cell layer, SGL = subgranular layer. Scale bar = 50 μm. N = 5, *p < 0.05, **p < 0.01



Supplementary Figure IV-1. Nissl staining of a coronal section of the adult rat brain on which the cannula was positioned. Arrows indicate the tract of cannulation from the pial surface to the lateral ventricle. CC = corpus callosum, LV = lateral ventricle, CP = choroid plexus. Scale bar = 200 μm.



Supplementary Figure IV-2. Traumatic brain injury alters the expression of VEGF. Quantitative analyses of VEGF expression as measured by enzyme-linked immunosorbent assay (ELISA). The amount of VEGF in the samples was within the range of 0.1 ~ 0.4 pg/μg total protein. VEGF expression in the ipsilateral dentate gyrus (DG) of TBI brain increased significantly 5 and 7 days post injury (dpi). Data represent averages of duplicated experiments. Error bars indicate SD. N = 5 for 5 dpi group and N = 4 for 7 dpi group. *$p < 0.05$, **$p < 0.01$, Ipsi = ipsilateral, contra = contralateral, Hpp = hippocampus, Ctx = cortex

CHAPTER V

DISCUSSION

The overall aim of this thesis work was to provide a better understanding about the role of intrinsic and extrinsic factors in *de novo* neurogenesis. The specific objectives in pursuit of the overall aim were a) to determine the role of an intrinsic factor, the nuclear regulatory protein AUF1 in the developing brain (Chapter II) and the adult brain (Chapter III), and b) to determine the role of an extrinsic factor, the growth factor VEGF and its receptor Flk1 in the adult injured brain (Chapter IV).

AUF1 and Neurogenesis in the Developing Brain

Data presented in Chapter II implicate AUF1 in regulating cortical neurogenesis in the developing brain through interactions with chromatin-remodeling molecules. This is important because chromatin remodeling plays a critical role in integrating the effects of genetic and epigenetic information in the developing brain from which different cell types originate even though their genetic background is basically the same.

AUF1 is expressed in proliferating neural stem / progenitors (and also in postmitotic differentiating cortical neurons), suggesting that AUF1 is involved in regulating proliferation of neural stem / progenitors and also differentiation of postmitotic neurons (Figure II-3). Based on its pattern of expression, AUF1 is likely expressed in self-renewing neural stem cells that undergo limited cycles of symmetrical cell divisions as well as neural precursor / progenitor cells

that are dividing asymmetrically [1]. In the E14 brain, AUF1+ cells in the VZ express Phospho-Histone H3 (PhH3), a marker of dividing cells indicating that AUF1 was expressed during the M phase of cell cycle (Figure II-3A-C). During M phase, inherited determinants of cell fate are localized to only one pole of the mother cell undergoing asymmetric cell division, thus only one of the daughter cells will inherit such a determinant [1,2]. Whether AUF1 belongs to such group of proteins and is only inherited by one daughter cell will need to be addressed in future experiments.

AUF1 proteins are co-expressed and interact with chromatin-remodeling molecules HDAC1 and MTA2 (Figures II-4 and II-5). This finding is potentially significant because chromatin modifications are an important component of epigenetic regulatory mechanisms. Epigenetics refers to alterations in gene expression that are controlled by heritable but potentially reversible modifications in DNA and / or chromatin structure without changes in the underlying DNA sequence of the organism [3]. Chromatin is a highly organized structure with genomic DNA and histone proteins. Chromatin structure is dynamic, modulated by enzymes and directly associated with gene expression. Histone acetyltransferases (HATs) are enzymes that acetylate specific lysine residues on histone proteins. Histone acetylation interferes with the interaction between the positively charged histone and the negatively charged DNA backbone. As a consequence of this, the transcription machinery can more freely access promoter regions and activate gene expression. Histone deacetylases (HDACs) on the other hand reverse the action of HAT. Besides this structural rearrangement of chromatin, other posttranslational modifications of histone by itself provide a specific signal for the activation of transcription machinery [4]. This combinatorial assortment of acetylation, methylation, and phosphorylation on histones has

been termed the 'histone code', which is critical for regulating cell type-specific gene expressions [5].

Altering histone acetylation modulates the effect of AUF1 on gene expression and the absence of AUF1 alters the composition of the chromatin-remodeling complex (Figures II-6 and II-7). These results suggest that AUF1 is involved in coordinating gene expression in proliferating neural precursor / progenitor cells and differentiating cortical neurons by recruiting chromatin-remodeling molecules. This is important because studies have shown that chromatin remodeling can play important roles at various stages of corticogenesis by coordinating the expression of multiple genes [6]. At the molecular level, they provide a permissive or restrictive chromatin environment for classical transcription factors during cellular differentiation. Little is known, however, about the identity of proteins directing their effect to specific genomic loci [7].

AUF1 and Neurogenesis in the Adult Brain

I demonstrated in the previous chapter that AUF1 is expressed by postmitotic differentiating young neurons in the E18 and neonatal rat brain (Figure II-3). Consistent with these results, I found that AUF1 is expressed by postmitotic differentiating young neurons in the DG of the adult rat brain (Figure III-1). This immunohistochemical analysis indicates that AUF1 is expressed in the similar stages of neuronal development, suggesting that AUF1 may have conserved role in the developing and adult brain. Interestingly, inhibition of histone deacetylase induces neuronal differentiation of multipotent adult neural progenitor cells [8]. As AUF1 is a multifunctional molecule, it can be involved in regulating neurogenesis via multiple ways. Whether AUF1 function on the adult hippocampal neurogenesis is also related in transcriptional

regulation [9] and / or recruiting chromatin remodeling molecules (Chapter II) and / or mRNA metabolism [10] will be addressed in future experiments.

I showed the size of the DG in AUF1 mutant is significantly reduced (Figure III-6A). Because the reduced size of the DG can be the result of decreased proliferation, I compared the number of proliferating cells in the SGL of AUF1 wild type and mutant animals. Although I observed decreased proliferation in the mutant brain (Figure III-7), the effect of AUF1 mutation on proliferation of adult hippocampal stem / progenitor cells appears to be dependent on age and / or on generation of mutant animals. My observation in the brain is in agreement with our collaborators' findings that the effect of AUF1 mutation on scarring in the skin and immune response to injuries are also age- and generation-dependent. These findings raise the interesting possibility that AUF1 may also alter telomerase activities and / or exert direct effects on the length of telomeres (Sadri et al., personal communication). It has been shown *in vitro* that the p42 isoform of AUF1 binds single-stranded telomere DNA and is involved in telomere maintenance [11,12].

I showed the number of Prox1+ cells scattered outside of the boundary of the DG increased by age and the difference between $AUF1^{+/+}$ vs. $AUF1^{-/-}$ animals after adjusting for age was statistically significant (Figure III-6B). New neurons are born in the SGL and migrate into the GCL. The increased number of Prox1+ postmitotic granule cells scattered outside of the boundary of the DG can be the result of impaired migration process. Our preliminary microarray data analyzing the transciptome of AUF1 mutant brains indeed showed that the expression levels of genes associated with cell-adhesion and migration are altered in the mutant animals (data not shown). These molecular abnormalities may contribute to the altered cytoarchitecture of the DG in AUF1 mutant.

VEGF and Neurogenesis in the Adult Dentate Gyrus

The data presented in Chapter IV suggest that VEGF is an important extrinsic determinant of *de novo* neurogenesis in the injured adult brain and that VEGF signaling is likely involved in regulating the differentiation / survival of *de novo* granule neurons. This finding can be potentially important because it suggests that VEGF signaling can influence *de novo* neurogenesis at a specific stage.

I found that VEGF expression is only elevated in the ipsilateral DG, but not in the ipsilateral hippocampus (minus DG) or the ipsilateral parietal cortex of the injured brain (Figure IV-3). As my pilot studies using Fluoro-Jade B histology have shown, these brain regions have suffered significant neuronal losses after trauma (Figure V-1), yet the hippocampus and the cortex do not have elevated VEGF expression. Transiently elevated expression of VEGF restricted to the DG has been reported after systemic hypoxia and electroconvulsive seizures [13,14]. The mechanism of how insults to the entire brain or multiple brain regions result in a signaling specific to the DG is currently not understood. An attractive hypothesis is that hemorrhage and hypoperfusion and / or alterations in vascular integrity change local blood flow, resulting in very localized hypoxia and / or ischemia [15]. Hypoxia is a well known inducer of VEGF expression by activation of VEGF gene transcription through hypoxia-inducible factor 1 (HIF-1) [16].

Flk1 is expressed in neuroblasts / young neurons and postmitotic granule cells in the adult DG (Figure IV-2). Based on the proposed cell types in adult hippocampal neurogenesis, cells expressing Flk1 are likely to belong to developmental stages 3 to 6 [17] (see also Figure I-3). The cell types in these stages are lineage determined, have limited self-renewal, are postmitotic,

and are undergoing differentiation. This suggests that in adult hippocampal neurogenesis, VEGF is involved in mediating the differentiation / survival process of young neurons rather than proliferation of neural stem / progenitor cells. I observed a significantly higher number of *de novo* neurons (Prox1+/BrdU+) after VEGF infusion than in the control group, whereas the number of proliferating (BrdU+) cells was not increased significantly (Figure IV-5). The observed net increase in the numbers of *de novo* neurons after exogenous VEGF treatment in the injured brain can be the result of increased survival and / or a decrease in the number of apoptotic cells. This finding suggests that VEGF contributes to enhanced hippocampal neurogenesis after TBI through neuroprotection rather than proliferation of neural progenitor cells.

Flk1 is a membrane bound tyrosine kinase whose activation stimulates various intracellular signal transduction pathways such as the PI3K/Akt and MEK/ERK pathways [18,19]. For example, the PI3K/Akt pathway mediates neuroprotective function of VEGF [20], while the MEK/ERK pathway influences proliferation of retinal progenitors [21]. For the neurogenic effect of VEGF, VEGF can act directly on Flk1-expressing neural progenitor cells and/or differentiating young neurons. It has been shown that VEGF/Flk1 contributes to neurogenesis rather than to angiogenesis in the DG after transient forebrain ischemia [22]. Furthermore, in the avascular chicken retina, VEGF acts through the Flk1 present on retinal progenitors to influence cell proliferation and commitment [21]. Although these studies support the idea that VEGF exerts its effect directly on Flk1 expressing neural progenitors and young neurons, VEGF can exert its effects on neurogenesis through other non-neuronal cell types in the brain. It should be noted that an intimate and symbiotic but not fully understood relationship between endothelial cells and neural stem cells in the “neurogenic niche” of the hippocampus

exists [23]. Endothelial cells provide various signals that can regulate the rate of proliferation of neural stem cells and the survival of *de novo* neurons in the DG [24,25]. Accordingly, VEGF can activate vascular endothelial cells and / or induce *de novo* angiogenesis, which in turns support *de novo* neurogenesis. Another possibility is that upregulated VEGF following injury to the brain activates astrocytes that in turn release additional factors that affect neurogenesis [26,27].

I found that chronic treatment with the Flk1 inhibitor SU5416 failed to reduce the number of *de novo* neurons and proliferating cells below control values in the injured adult brain. Because SU5416 was delivered into the ipsilateral side of the injured brain for two weeks, upregulated expression of endogenous VEGF following TBI might interfere with the action of SU5416. Alternatively, there may be another receptor and/or Flk1-independent mechanism involved in VEGF signaling in the injured brain.

VEGF and Neurogenesis in the Developing Brain

Using primary embryonic neuronal culture model, I showed that VEGF has proliferative and antiapoptotic effects that are likely mediated by VEGF receptor Flk1 (Appendix Figure 1, 2). These findings are consistent with the results from previous studies also showing that VEGF has both mitotic and neurotrophic functions. VEGF treatment of mouse embryonic neuronal cultures resulted in an increased number of BrdU labeled cells [28]. A separate study showed that VEGF treatment of embryonic mesencephalic explant cultures increased survival and neuritic outgrowth of dopaminergic [29] and cortical neurons [30]. It also has been shown that both the proliferative and the neurotrophic effect of VEGF are mediated by Flk1 [28,30]. My findings along with the results from others suggest that VEGF function and likely regulatory mechanism on neurogenesis are, at least in part, conserved between the developing and adult brain.

Functional Relevance of *de novo* Neurogenesis after Brain Injury

Although I did not show that the increased number of *de novo* neurons in the DG after TBI is directly linked to a hippocampus-dependent functional outcome, there is supporting evidence that enhanced neurogenesis after TBI contributes to the functional recovery. Because of the BrdU infusion paradigm (BrdU was infused for 2 weeks, followed by a 2-week survival period) the detected *de novo* neurons in this thesis work are up to 4 weeks old. Because a significant portion of newborn cells die within the first few days after birth, new granule cells that express neuronal markers have an increased chance of survival for longer periods and become functional, as previously demonstrated [31]. The granule cells of the DG are part of the major afferent pathway in the hippocampus. As a consequence, newborn neurons contribute significantly to various hippocampus-related functions such as learning and memory, as positive correlation occurs between learning and *de novo* neurogenesis [32,33]. Conversely, inhibition of neurogenesis, either by application of cytostatic drugs or by local irradiation, impairs hippocampus-dependent learning tasks [34,35]. Furthermore, adult neurogenesis may play a role in maintaining neuropsychiatric functions other than learning and memory [36]. For example, dysfunction of adult hippocampal *de novo* neurogenesis is closely related in major depression [36-38]. The observed behavioral effects of chronic treatment with antidepressants and antipsychotic drugs may be mediated through stimulating of hippocampal *de novo* neurogenesis [39,40]. These and other examples illustrate that sustained *de novo* hippocampal neurogenesis in the adult DG is critical for maintaining normal neuropsychiatric functions.

Brain injury-induce neurogenesis is closely associated with functional recovery. Enhanced neuronal recruitment following neuronal death in the high vocal center of adult

songbirds is related to song behavior [41]. New neurons generated in the DG of adult rat brain following TBI are capable of receiving synaptic input and projecting axons to the CA3 region of the hippocampus [42]. Importantly, this anatomical integration is temporally associated with innate cognitive recovery [42,43]. Moreover, elimination of cell proliferation in the DG after TBI reduces cognitive recovery in adult rats [44]. These results suggest that TBI-induced neurogenesis is crucial for functional recovery from the initial cognitive impairment and memory deficit after TBI.

It should be noted here that increased *de novo* neurogenesis after brain injury is not always beneficial. For example, in the pilocarpine-induced seizure model, the increased number of new granule cells in the DG migrates to aberrant locations and thus may worsen the outcome [45].

Interaction between Intrinsic and Extrinsic Factors

The plasma membrane separates a cell from the surrounding environment and extrinsic factors from intrinsic factors as well. Only small lipophilic molecules such as steroid hormone glucocorticoids can diffuse into the cytoplasm through the plasma membrane. Within the cell, a glucocorticoid binds to its receptor that translocates to the nucleus and recognizes a specific DNA element. As impermeable to the plasma membrane, hydrophilic molecules interact with membrane bound receptors. Some molecules such as ions are physically transmitted from the extracellular space to the cytoplasm via membrane bound channels. The other molecules including protein growth factors only transmit a signal by converting receptors from an inactive to an active form. The original signals are transduced to the nucleus by various second messengers through signal transduction pathways. Accordingly extrinsic factors are able to

interact with intrinsic factors. On the other hand, intrinsic factors can modulate the extent of extrinsic factors, for example the copy number of EGFR can affect proliferation, migration, and fate determination of neural stem / progenitor cells [46]. For functional neurogenesis all intrinsic and extrinsic cues should be coordinated and integrated properly.

The function of AUF1 can be modulated by extrinsic factors. *In vitro* experiments showed that the activity of AUF1 p40 isoform is regulated through phosphorylation on specific Ser residues by protein kinase A (PKA) and glycogen synthase kinse 3β (GSK3β) [47]. Whether or not AUF1 can directly or indirectly regulate the expression of VEGF is currently not known. It has been shown that VEGF expression is regulated at the posttranscriptional level through altering the stability of its mRNA [48]. The structure of VEGF mRNA has the common feature of labile mRNA. The 3'-UTR of VEGF mRNA contains multiple copies of AREs that RNA binding proteins, such as AUF1, can interact with. Several proteins have been shown to interact with the VEGF mRNA 3'-UTR such as Hu-antigen R (HuR), a member of the embryonic lethal abnormal visual (ELAV) family [49] and heterogeneous nuclear ribonuclear protein L (hnRNPL), a member of the hnRNP family [50]. Interestingly, it has been shown that HuR and AUF1 bind to many common AREs and exert opposite function on target mRNA stability [51]. AUF1, also known as hnRNPD, was originally identified as a protein that binds to AREs of the 3' UTR of mRNAs and acts as a regulator of mRNA stability [15]. Moreover, VEGF expression is transiently elevated in the DG under pathological conditions [13,14] and AUF1 expression is observed in the DG (Figure III-1). These information provide the rationale for future experiments aimed at investigating the role of AUF1 in regulating VEGF gene expression through altering its mRNA stability.

Summary and Future Works

I have shown that AUF1 is involved in regulating cortical neurogenesis in the developing brain through interactions with chromatin-remodeling molecules, which is a novel role for this already multifunctional molecule. In addition, I have shown experimental evidence that AUF is involved in adult hippocampal neurogenesis. Future experiments will determine whether its action in the adult hippocampus also involves chromatin remodeling or some other regulatory mechanism(s).

I have shown that VEGF is an important mediator of *de novo* neurogenesis in the injured adult brain. Future experiments will need to identify the molecular regulatory mechanism through which VEGF affects proliferation and differentiation / survival.

In summary, my studies provided novel information about the regulation of *de novo* neurogenesis in the developing and in the injured adult brain. This is important because a better understanding about the identity of molecules and their regulatory mechanisms will help to identify the genetic and environmental risk factors leading to developmental brain disorders and also will help to develop treatment options to alleviate the consequences of brain injury.

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CHAPTER V Figure

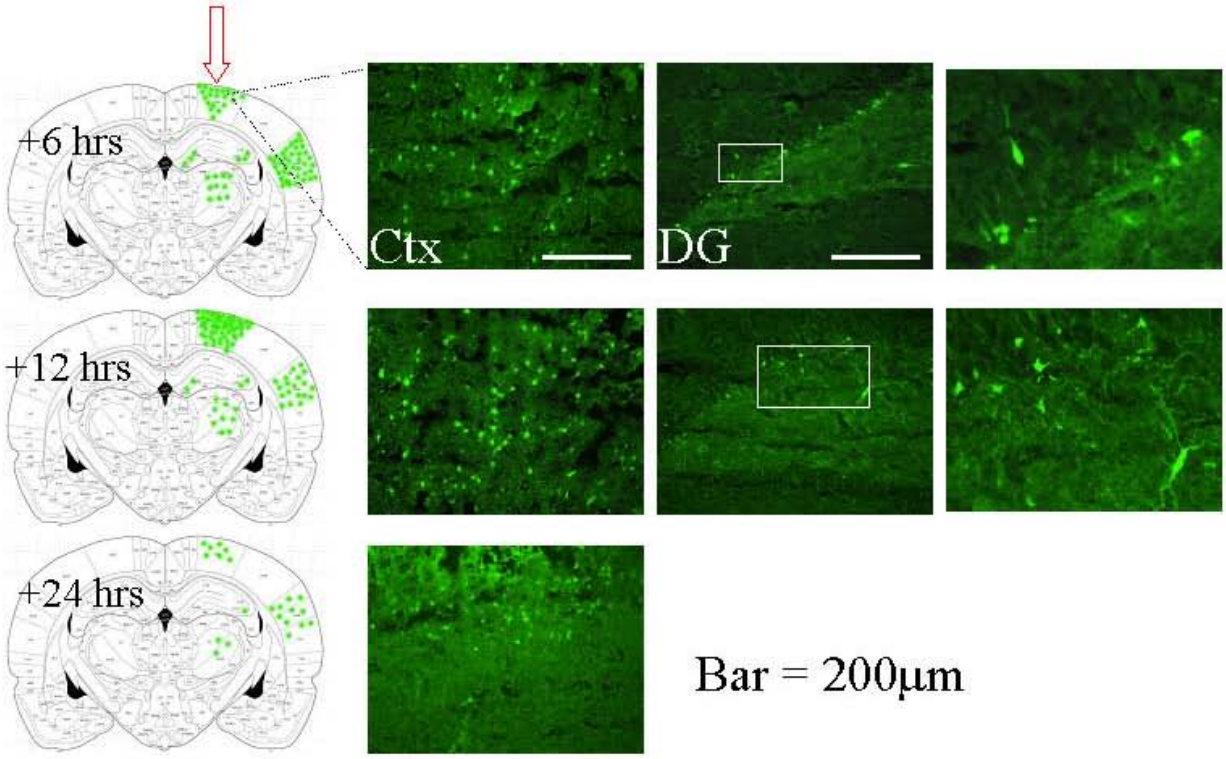


Figure V-1. The spatial and temporal distribution of FJB+ cells after TBI. Red arrow indicates the impact site. Insets in the middle column are enlarged to the images in the right column. Ctx = cortex, DG = dentate gyrus.

APPENDIX

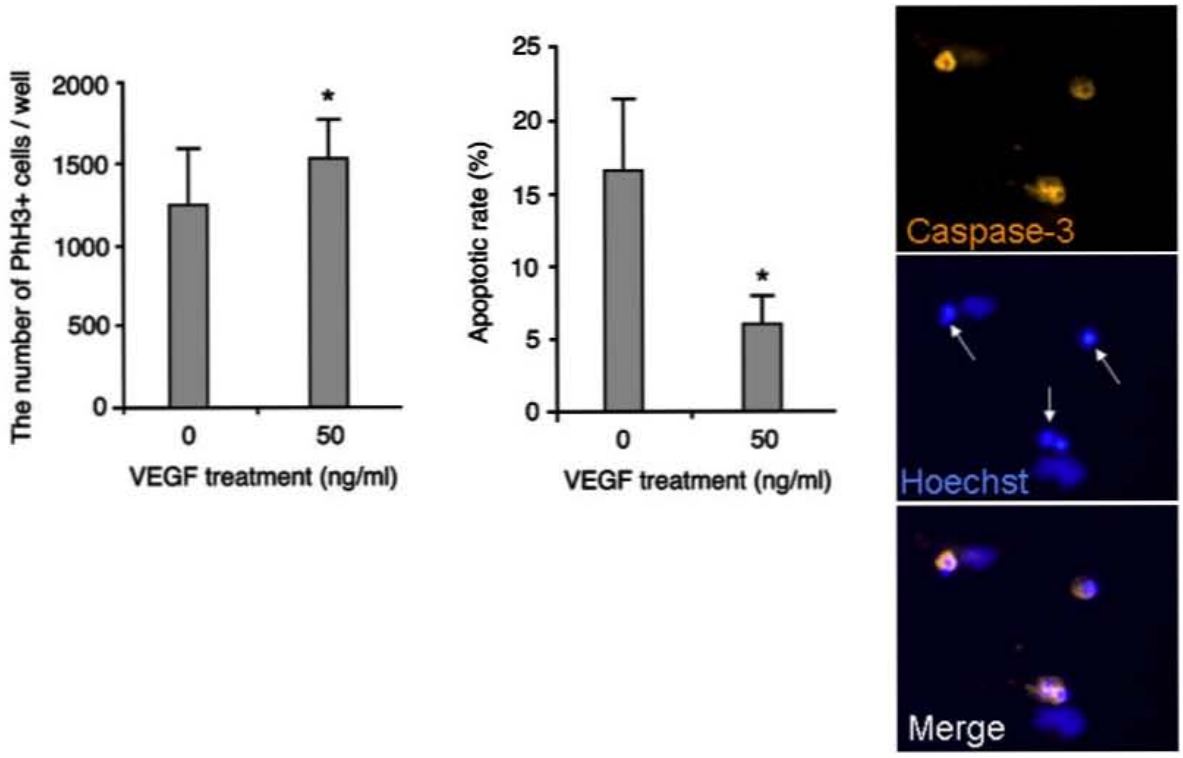
THE ROLE OF VEGF ON NEUROGENESIS IN THE DEVELOPING BRAIN

I showed that VEGF signaling is a positive factor of *de novo* hippocampal neurogenesis in the injured adult brain. To investigate the role of VEGF on neurogenesis in the developing brain, I performed pilot experiments.

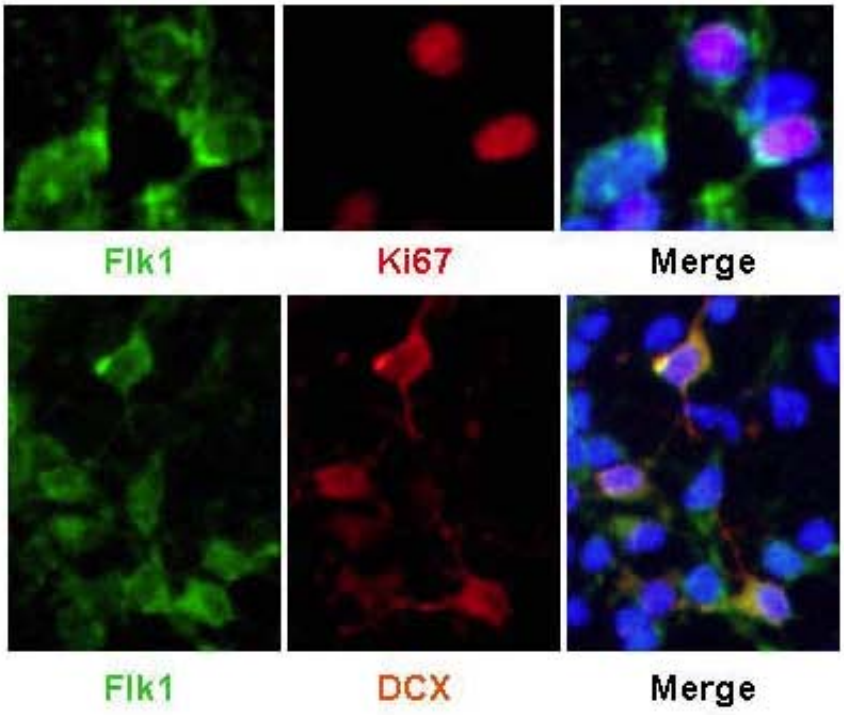
I microdissected telencephalic vesicles were from E13.5 rat brains and dissociated cells using the papain dissociation system (Worthington Biochemical, Lakewood, NJ). I suspended cells in culture medium and plated onto 24-well culture plates at a density of 10^5 cells per well. I maintained cultures at a 37°C CO2 incubator. On the next day, I changed a half of the medium and treated cells with 50 ng/ml VEGF (rat recombinant $VEGF_{164}$, Sigma). I treated control cultures only with saline. 24 hours later, I fixed cultures with 4% paraformaldehyde and processed for immunocytochemistry. To determine VEGF effect on proliferation, I quantified total number of Phospho-Histone H3+ cells in each well. I analyzed four wells both from VEGF treated and control group. To determine VEGF effect on apoptosis, I determined the apoptotic rate (cleaved-caspase-3+ cells divided by Hoechst 33342+ cells). For this, I selected five view fields (12, 3, 6, 9 o'clock direction and the center) from each of four wells, both from VEGF treated and control group. Cleaved-caspase-3 is a marker for apoptotic cells and Hoechst dye was used for nuclear staining. Condensed nucleus is a typical feature of apoptotic cells which are matched with caspase staining. In this *in vitro* model of the developing brain, VEGF stimulated proliferation of neural precursor cells and reduced apoptosis (Appendix Figure 1).

To determine whether the observed VEGF effects were mediated by Flk1, I characterized the phenotype of Flk1 expressing cells in the embryonic neuronal cultures by immunohistochemistry. Sister cultures of the previous VEGF treatment experiment were fixed with 4% paraformaldehyde and processed for double immunofluorescence. A subset of Flk1+ cells coexpressed Ki67, a marker of proliferating cells, and double cortin (DCX), a marker of neuroblast / young neurons (Appendix Figure 2). These results show that Flk1 is expressed in proliferating cells and young neurons in this *in vitro* model of the developing brain, suggesting that observed proliferative and antiapoptotic effects of VEGF are likely mediated by VEGF receptor Flk1.

Appendix Figures



Appendix Figure 1. VEGF has proliferative and antiapoptotic effect on primary embryonic neuronal cultures. Telencephalic vesicles were microdissected from E13.5 rat brains. Cells were dissociated and plated onto 24-well culture plates. On the next day, cells were treated with 50 ng/ml VEGF (rat recombinant VEGF164). Control cultures were treated only with saline. 24 hours later, cultures were fixed and processed for immunohistochemistry. To determine VEGF effect on proliferation, total number of Phospho-Histone H3+ (PhH3+) cells was quantified. To determine VEGF effect on apoptosis, the apoptotic rate (cleaved-caspase-3+ cells divided by Hoechst 33342+ cells) was analyzed. Cleaved-caspase-3 is a marker for apoptotic cells and Hoechst dye was used for nuclear staining. Condensed nucleus is a typical feature of apoptotic cells which are matched with caspase staining. N = 4 (Four wells both from VEGF-treated and saline-treated group were used), *$p<0.05$



Appendix Figure 2. Flk1 expressing cells in the primary embryonic neuronal culture express Ki67 and DCX. To determine whether the observed VEGF effects were possibly mediated by Flk1, the phenotype of Flk1 expressing cells was analyzed. Sister cultures of the previous VEGF treatment experiment were processed for double immunofluorescence. A subset of Flk1+ cells coexpressed Ki67, a marker of proliferating cells and double cortin (DCX), a marker of neuroblast / young neurons. Cells were counterstained with Hoechst dye (blue) in the merged images.